peptide and a transmembrane/cytoplasmic tail. The all or a portion of the auto-antigen is processed by endosomes as a result of these functional connections. (Claim 54.)

The invention is also drawn to a virus that infects human APCs. The virus comprises a polynucleotide that encodes all or a portion of an auto-antigen to which the autoimmune disease patient's antigen-specific T cells respond. (Claim 58.)

The Amendments

Claim 54 has been amended to recite "transfected" instead of "tranfected." The amendment merely corrects the spelling of transfected and does narrow the scope of the claim. It also introduces no new matter and does not require a new search. The amendment has not previously been made because claim 54 is a new claim, added in the prior response to Office Action, and applicant was first aware of the incorrect spelling at this time.

Claim 58 has been amended to recite "an auto-immune disease" instead of "the auto-immune disease." The amendment corrects antecedent basis of auto-immune disease, which is not recited in a prior occurrence in the claim. Thus the amendment does not narrow the scope of the claim. It also does not introduce new matter or require a new search. The amendment was not previously made because claim 58 is a new claim, added in the prior response to Office Action, and applicant was first aware of the improper recitation at this time.

The Rejection of Claims 41-67 Under 35 U.S.C. § 112

Claims 41-67 are rejected under 35 U.S.C. § 112 for lack of enablement. Applicant respectfully traverses.

The Patent Office makes this rejection, in part, by asserting reasons to doubt applicant's disclosure. The MPEP § 2164.04 sets forth that in making an enablement rejection, the PTO must accept applicant's specification as true, unless there are reasons to doubt it. "A specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as being in compliance with the enablement requirement of 35 U.S.C. 112, first paragraph, unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support." If the Patent Office finds reason to doubt applicant's disclosure, the MPEP also sets forth that the PTO must provide sound scientific reasoning or evidence to support its case. Should the Patent Office find a lack of enablement, it must "explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement." The PTO has provided numerous reasons why it doubts applicant's supporting disclosure, but does not provide evidence or reasoning to support all of its doubts of the accuracy of applicant's statements.

The Office Action doubts that the claims are enabled because applicant's working examples are *in vitro*, which may not reflect the results achieved *in vivo*, or in a patient. The Office Action asserts, "APC cells transfected with a nucleic acid are eventually introduced into a patient and therefore, subjected to an environment in the patient which is entirely different to that in cell culture. Therefore, even though some *ex vivo* experiments are in clinical trials, *ex vivo* gene therapy is not enabled across the board." (Paper 15, page 3, lines 12-14.) Applicant has supplied *in vitro* experimental evidence to demonstrate the instant claims are enabled. The

specification provides data that "both FasL and anti-Fas antibody dramatically inhibited T cells that were stimulated either by endogenously or by exogenously presented AchR." (Page 15, line 23 through page 16, line 2.) The specification also discloses that "CTLA4Ig inhibited AchR-specific T cell proliferation by ~70%, which is consistent with previous results *in vitro*." (Page 18, lines 9-10.) Thus, applicant has demonstrated his approach works *in vitro*. The fact that some *ex vivo* gene therapies are in clinical trials, coupled with the experimental data disclosed in the specification supports enablement of the claims. The Patent Office need not doubt the claims are enabled.

The Office Action cites Crystal as teaching that results of *ex vivo* clinical trials have been inconsistent. (Paper 15, page 3, lines 15-17.) The Office Action specifically refers to an example in Crystal in which two children afflicted with ADA received autologous T cells which were modified *ex vivo* with normal ADA cDNA. The Office Action asserts that the results observed in that trial were inconsistent because, "The normal ADA+ cells varyied between 0.1-60%." (Paper 15, page 3, line 19.) Although the number of ADA+ cells varied in this example between 0.1 and 60%, there were ADA+ cells at all times in each of the patients. The Office Action also asserts that Crystal teaches that *ex vivo* clinical trials are inconsistent by stating that:

> Additionally, even though successful transfer of the ADA gene to the epithelium is observed successful expression is only observed in 5% of cells.

Paper 15, page 3, lines 20-21. This reference to Crystal improperly characterizes the data. Crystal does not teach that the ADA cDNA is expressed in only 5% of epithelial cells. Crystal teaches that CFTR cDNA is transferred to the respiratory epithelium in about 5% of cells.

However, the CFTR trial was an *in vivo* cystic fibrosis gene therapy trial and therefore not relevant to *ex vivo* gene therapy. (See Crystal page 409, column 1, lines 29-35; see also table 2.)

Furthermore, Crystal teaches that *ex vivo* gene therapy is generally successful as a treatment for a variety of genetic disorders. Crystal states:

> Retrovirus vectors also have been used to transfer therapeutic genes ex vivo, with success demonstrated by the fact that the modified cells exhibit their altered phenotype in vivo for up to 36 months (Table 1).

Emphasis added, page 405, third column, lines 43-47. Crystal also teaches specific examples of successful gene transfer as a result of *ex vivo* gene therapy:

> Successful gene transfer has been demonstrated in cells recovered form children with adenosine deaminase (ADA) deficiency after transfer of the normal ADA complementary DNA (cDNA) to autologous T cells, cord blood, and placental cells; from individuals with solid tumors after transfer of cytokine cDNAs in autologous vaccine strategies to fibroblasts, TILs, or tumor cells; from individuals with familial hypercholesterolemia after transfer of the low-density lipoprotein (LDL) receptor cDNA to autologous hepatocytes; from HIV^+ siblings after transfer of a chimeric T cell receptor cDNA to blood T cells of a twin; and from individuals with tumors who received autologous marrow transplants after transfer of the multidrug resistance 1 cDNA to autologous blood CD34+ stem cells.

Page 405, third column, line 52 through page 406, first column, line 7, citations omitted. Clearly, Crystal teaches that *ex vivo* gene therapy had been successful. The data reviewed by Crystal supports rather than detracts from the enablement of the claimed invention. The successful clinical trials reviewed by Crystal combined with the *in vitro* data disclosed by applicant in the specification demonstrate that those of skill in the art would have had a reasonable expectation of success in practicing the invention without recourse to undue experimentation.

The Office Action also alleges that the claims are not enabled because it doubts that applicant's disclosure of *in vitro* data is indicative of *in vivo* clinical therapy when APCs are removed from a patient and used according to the methods of the claims. The Office Action asserts "it is not clear that the APC cells removed from the patient and transfected with a polynucleotide encoding at least a portion of AchR and FasL and FADD will simulate that which occurs in a lymphoma cell line transfected ex vivo." (Paper 15, page 4, lines 10-13.) Applicant respectfully traverses. The Office Action offers no evidence or sound scientific reasoning why APCs transfected *ex vivo* should perform in the patient differently than *in vitro*. Moreover, Crystal provides evidence that the transfected or transformed cells will perform as expected in a patient. Crystal reviews several studies that demonstrate successful function of cells which were transfected *ex vivo*.

> In one ex vivo study a retrovirus vector was used to transfer the interleukin-4 (IL-4) cDNA to autologous fibroblasts. The cells were then irradiated and implanted subcutaneously in the donor together with irradiated, unmodified, autologous tumor cells. In some recipients, this evoked infiltration with $CD3^+$ T cells and tumor-specific $CD4^+$ T cells at the immunization site, as well as enhanced expression of cell adhesion molecules on capillary endothelium. In another trial, autologous neuroblastoma cells modified ex vivo with a retrovirus to contain the IL-2 cDNA were lethally irradiated and implanted subcutaneously. In some individuals, this evoked systemic augmentation of $CD16^+$ natural killer cells and tumor-specific $CD8^+$ cytotoxic T cells and eosinophilia.

Page 408, first column, lines 27-45, citations omitted. In each study Crystal mentions, the implanted transfected cells initiate an immune response *in vivo*. The cells expressing IL-2 result in systemic augmentation of $CD16^+$ natural killer cells and tumor-specific $CD8^+$ cytotoxic T

cells. Crystal teaches that the immune responses are as would be expected, within the patient. Thus there is no reason for the Patent Office to doubt applicant's disclosure.

The Office Action voices concern that autoimmune specific T cells will be activated by the transfected or transformed APCs, rather than ablated. The Office Action asserts that "it is not clear that the APCs transfected with AchR, will inactivate the AchR specific T cells rather than stimulate them." (Paper 15, page 4, lines 13-14.) In fact, the method intends the T cells to be stimulated. It is the administration of a product that is detrimental to activated T cell proliferation which will prevent proliferation of the AchR specific T cells. The specification teaches that detrimental products such as CTLA4Ig, Fas ligand, and antibody to Fas, inhibit the proliferation of activated AchR specific T cells. "When stimulated in the presence of CTLA4Ig, the T cells are relatively inactivated; when stimulated in the presence of Fas ligand or antibody to Fas they undergo apoptosis and death." Page 9, lines 9-11.) Thus AchR does activate AchR specific T cells and such activation is required by the claims.

The Office Action further alleges that the state of the art is unpredictable and that applicant's *in vitro* data are insufficient to support the claims. The Office Action asserts that "one of skill in the art will not expect the results obtained from in vitro cell culture to be suggestive of ex vivo experimentation." (Paper 15, page 5, lines 3-4.) Applicant respectfully traverses. The Crystal reference, as addressed earlier, teaches numerous instances that demonstrate that *ex vivo* gene therapy works. Crystal teaches, "Taken together, the evidence is overwhelming, with successful human gene transfer having been demonstrated in 28 ex vivo and 10 in vivo studies (Table 1)." (Page 405, third column, lines 9-13, emphasis added.) Crystal teaches, in Tables 1 and 2, successful *ex vivo* gene therapy trials for ADA, LDLR, and various

types of tumors such as melanoma, neuroblastoma, lung cancer, and breast cancer. Thus one of ordinary skill in this art would find the results obtained from *in vitro* cell culture indicative of successful *ex vivo* methods. The state of the art supports the enablement of the claims. There is no basis for doubting applicant's supporting disclosure.

The Patent Office also questions whether the viral vector used to treat the patient with an autoimmune disease will elicit an immune response and render the claimed method ineffective. The Office Action asserts that "the introduction of a viral vector, unless completely gutted, will respond by eliciting an immune response opposing and contrary to the intent of the instant application." (Paper 15, page 5, line 22 through page 6, line 2.) The claimed method does not require that a viral vector be introduced directly into the patient. The claimed method recites that APCs are removed from a patient. A polynucleotide, which may be in a viral vector, is introduced into the APCs. The APCs are reintroduced into the patient. Thus, no viral vector is introduced directly into the patient. Furthermore, a viral vector is not necessarily used to introduce the polynucleotide into the APCs. The specification discloses that non-viral techniques can be used. (Page 7, line 22.) However, the introduction of a viral vector may occur when APCs that are transduced with a viral vector are implanted into an autoimmune disease patient. The specification describes that the viral vectors used in the invention do not have the ability to replicate, which would prevent viral spread and contain the immune response. The specification discloses that "it is preferred that the viruses be attenuated so that they do not replicate." (Page 8, line 1.) Typically those of skill in the art use defective viruses as gene therapy vectors to prevent the generation of any immune response.

The Office Action speculates that there is doubt as to whether AchR will simulate auto

antigens in any patient afflicted with any auto immune disorder. There is, however, no reason provided to doubt that autoimmune T cells will be activated by their autoantigens. Autoimmune T cells are by definition activated by their autoantigens. The specification discloses that "autologous antigen presenting cells (APCs) can be transfected or transduced to express, process, and properly present an antigen to antigen-specific T cells. Moreover, upon proper presentation, the antigen-specific T cells are activated." (Page 5, lines 15-20.) There is no reason provided by the Patent Office to doubt applicant's disclosure.

The Patent Office also doubts the enablement of the claimed method because there is allegedly no disclosure of what will happen in an autoimmune patient upon introduction of cells transduced or transfected with FasL. Office Action alleges that, "It is not clear how these transfected cells comprising FasL will behave in a patient with an autoimmune disorder." (Paper 15, page 6, lines 7-9.) The Patent Office, however, has offered no evidence or sound scientific reasoning that the transfected cells will not behave in a patient with an autoimmune disorder as they do *in vitro*. Applicant submits evidence with this response that the results of *ex vivo* gene therapy trials demonstrate that cells reintroduced into a patient yield the response that is expected according to *in vitro* studies. See Fu et al. (Tab C), Malech et al. (Tab B), and the examples listed in Tables 1 and 2 of Crystal, previously discussed. There is no reason provided by the Patent Office to doubt that the cells will behave in a patient as taught.

The Patent Office questions the enablement of the claims because not all the claims require that cells transfected or transduced with Fas ligand are also transduced or transfected with the protective molecule FADD. The Office Action points out that not all claims recite the use of FADD for protection of introduced cells against destruction. "It is not clear how these

transfected cells comprising FasL will behave in a patient with an autoimmune disorder." (Paper 15, page 6, lines 7-9.) The specification discloses that "the expression of Fas ligand by a cell can be suicidal." (Emphasis added, page 7, lines 15-16.) Thus the APCs may or may not survive without additional expression of a protective molecule. The APCs that comprise FasL, but not FADD, may comprise a different protective molecule. The specification discloses that there are protective molecules other than FADD and that "these polypeptides are known in the art to protect expressing cells from the pro-apoptotic effects of Fas ligand." (Page 7, lines 18-19.) Thus, the lack of FADD expression does not necessarily result in death of APCs expressing Fas. The Office Action provides no evidence that cells comprising FasL and not FADD would not survive.

The Office Action also doubts the enablement of the claimed method because it alleges that applicant's *in vitro* disclosure using AchR activated T cells as an example of autoimmune disease may not apply to other auto antigens in patients with autoimmune disease. The Office Action asserts that "it is not clear that this one example wherein the epitopes of an α-subunit of a torpedo acetyl choline receptor are used *in vitro* experiments represent all autoantigens of patients with autoimmune disorders." (Paper 15, page 6, lines 11-13.) The Office Action again provides no evidence or reasoning for this allegation. There is no reason to doubt that such a treatment will work for autoantigens of patients with autoimmune disorders. The specification discloses that "autologous antigen presenting cells (APCs) can be transfected or transduced to express, process, and properly present an antigen to antigen-specific T cells. Moreover, upon proper presentation, the antigen-specific T cells activated. Activation of the selected class of antigen-specific T cells permits this class to be distinguished from other T cells and for them to

be selectively targeted for ablation." (Page 5, lines 15-20.) The Patent Office has offered no evidence to doubt applicant's disclosure. Speculation is not sufficient to shift the burden of proof to the applicant.

The Office Action also questions whether "the one example (FasL) used in the in vitro experiments as a product that is detrimental to activated T cells [is] representative of all products detrimental to activated T cells as claimed?" (Paper 15, page 6, lines 14-16.) Again the Patent Office merely speculates and muses. The specification discloses several examples of products that are detrimental to T cells, with evidence to support their deleterious effects. The specification describes "[w]hen stimulated in the presence of CTLA4Ig, the T cells are relatively inactivated; when stimulated in the presence of Fas ligand or antibody to Fas they undergo apoptosis and death." (Page 9, lines 9-11.) Thus APCs that are transfected with AchR epitopes are inactivated by CTLA4Ig, and induced to undergo apoptosis by Fas ligand or antibody to Fas. Further support for the effect of these products is described in the examples.

> To examine the ability of these APCs to be used in inactivate- rather than stimulate the AchR-specific cells, endogenous stimulation of the target T cells by the transfected APCs was combined with simultaneous treatment with CTLA4Ig (to block costimulation). Endogenous stimulation by transfected A20 cells in the presence of 50 μg/ml of CTLA4Ig resulted in 70% inhibition of stimulation (Fig. 3), which is consistent with results of previous studies of in vitro effects of CTLA4Ig on AchR-specific T cell stimulation.

Page 15, lines 7-13. Thus the specification describes experimental evidence as to the fate of AchR specific T cells when exposed to the detrimental product CTLA4Ig and AchR specific APCs. The specification further describes experimental evidence regarding the fate of AchR

specific T cells when exposed to the detrimental products FasL or antibody to Fas and AchR specific APCs.

The specification discloses,

> The ability of Fas ligand (FasL) and antibody to Fas to induce killing of AchR-specific T cells that were stimulated by transfected APCs (Fig. 4) was tested. In order to allow the transfected APCs to complete their stimulation of the T cells, and to avoid possible interference with the APCs, FasL or anti-Fas antibody were first added 72 hrs after initiation of the cultures. Antibody to Fas resulted in virtually 100% inhibition of T cell proliferation, and treatment with soluble hFasL produced 70 to >90% inhibition.

Page 15, lines 14-19. Thus the effect of the detrimental products FasL and anti-Fas antibody were tested as well. Applicant has demonstrated the effect of three representative examples of detrimental products on the stimulated T cells. Thus there is no reason provided to doubt that detrimental "products" would function as taught for FasL.

Claims 54-57 and 66 are drawn to antigen presenting cells and claims 58-64 and 67 are drawn to viruses that infect antigen-presenting cells. These claims have utility other than in the claimed methods of treating of patients with autoimmune disorders. The APCs and viruses can be used to screen and identify treatments that are effective in arresting antigen-specific T cell growth or in eliminating antigen-specific T cells. The specification discloses that, "Such antigen-specific T cell activation provides a model system in which drugs and treatments can be screened to identify those which are effective in arresting growth of or eliminating the antigen-specific T cells." (Page 5, lines 21-23.) The specification also discloses that in the model system, the "genes which encode such agents can be supplied to the antigen presenting cells, such that upon interaction of the antigen presenting cells and the antigen-specific T cells, the latter cells can be both activated and ablated." (Page 6, lines 6-8.) Thus, the APCs may also be

used to determine further agents that can be used to inactivate or ablate antigen-specific T cells in an autoimmune disease patient. The claimed viruses may be used to transduce those APC cells. The Patent Office has asserted no reasons to doubt that those of skill in the art could make and use the claimed APCs and viruses.

The Patent Office has alleged that the claims are not enabled because various aspects of the invention are not taught. However, a patent need not teach, and preferably omits, what is well known in the art. *In re Buchner*, 929 F.2d. 660, 661 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384 (Fed. Cir. 1986); and *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrck Co.*, 730 F.2d 1452, 1463 (Fed. Cir. 1984). The specification, the prior art, or both teach each of the deficiencies asserted by the Patent Office.

The Office Action questions how one would know how many transduced or transfected APCs are to be administered to an autoimmune patient taking into consideration that the patient has an auto immune disorder and already has activated T cells. (Paper 15, page 4, lines 14-16.) Applicant respectfully traverses. The number of APCs administered to a patient with an autoimmune disease can be readily determined by one of skill in the art using standard titration procedures. Moreover, *ex vivo* clinical trials in patients with immune diseases had already been conducted prior to the filing of the instant application. Malech et al. teach *ex vivo* gene therapy to treat patients with the $p47^{phox}$ deficiency form of chronic granulomatous disease, as described previously. (Tab B.) Chronic granulomatous disease is "a rare inherited disorder of phagocytes associated with recurrent life-threatening infections." (Page 12133, first column, lines 1-3 after

the abstract.) Malech et al. teach, "The total number of cells infused ranged from 0.1 to 4.7 x 10^6 cells per kg (Table 1)." (Page 12135, column 1, lines 5-7.) See pages 12133 to 12134 in the Materials and Methods. One of skill in the art, at the time the application was filed, could have readily determined how many T cells to administer to a patient with an autoimmune disease without undue experimentation, relying on the prior art and the level of skill in the art.

The Office Action also alleges that the claims are not enabled because the specification does not teach all the autoantigens that can be used in the claims, and does not teach whether any autoantigen will elicit the same response in the autoantigen specific T cells as the response observed with AChR. The Office Action asserts that the "specification does not teach one where and how to obtain the DNA encoding autoantigens and if the AchR will simulate autoantigens in any patient afflicted with any autoimmune disorder." (Paper 15, page 4, lines 16-18.) Applicant respectfully traverses. DNA encoding autoantigens are well known to those of skill in the art. A patent need not teach, and preferably omits, what is well known in the art. The specification discloses that any auto-antigen known in the art may be used to treat a patient with an auto-immune disorder. "This technique can be used with any auto-immune disease in which the inciting auto-antigen is known and for which the coding sequence is available." (Page 6, lines 9-10.) Nucleotide and polypeptide sequences of human autoantigens retrieved from the NCBI database are shown at Tabs D through N. Each was entered before December 3, 1997, the date of the provisional application upon which priority is claimed. Accession number U01882 discloses the sequence for the human SS-A/Ro autoantigen which is associated with the autoimmune response in mothers whose children have manifestations of neonatal lupus (Tab D). Accession number D86115 discloses the nucleotide sequence for the human HC21Exc65 exon,

an auto antigen in polyglandular disease type I (Tab E). Accession number X56687 discloses the nucleotide sequence for the human NOR-90 auto antigen, a nucleolar transcription factor that is a novel target for human autoimmune response (Tab F). Accession number U26593 discloses the nucleotide sequence for the human diabetes mellitus I auto antigen ICAp69 (Tab G). Accession number X54162 discloses the nucleotide sequence for a novel 64-kDa auto antigen recognized patients with autoimmune thyroid disease (Tab H). Accession number X16163 discloses the nucleotide sequence for a human systemic lupus erythematosus autoimmune antigen (Tab I). Accession number X69936 discloses the nucleotide sequence for the human glutamic acid decarboxylase auto antigen of insulin-dependent diabetes mellitus patients (Tab J). Accession number J04977 discloses the nucleotide sequence for the human Ku auto antigen (Tab K). Accession number J03798 discloses the nucleotide sequence for the human small nuclear ribonucleoprotein Sm-D, an auto antigen of rheumatic disease systemic lupus erythematosus (Tab L). Accession number M24499 discloses the nucleotide sequence for the human autoantigen cytochrome P450db1, of idiopathic autoimmune hepatitis (Tab M). Accession number M28639 discloses the nucleotide sequence of human autoimmune thyroid disease-related antigen mRNA (Tab N). Thus one of skill in the art could readily use known or identify additional nucleotide sequences of autoantigens for use in the method of ablating autoantigen specific T cells as recited in the claims.

The Office Action further asserts that the specification lacks guidance as to how one is to obtain enough APCs from the patient. Applicant respectfully traverses. The specification clearly guides one of skill in the art how to obtain APCs.

> Antigen presenting cells can be withdrawn from an auto-immune disease patient, such as a myasthenia gravis patient according to

> techniques well known in the art. They can be found in the blood as well as in the bone marrow. In one embodiment of the invention B cells are purified from the blood and used as the preparation of antigen presenting cells.

Page 6, lines 11-15. The specification discloses that the APCs may be obtained from blood or bone marrow. In addition, one of skill in the art would know how to isolate these cells. For example, see McLellan et al. (J. Immunol. Methods, Vol. 184, 1995, 81-89; tab O) for an example of isolation of APCs from blood. McLellan et al. teach isolation of dendritic cells from the blood. It is not necessary to disclose those methods that are well known to those of skill in the art. If the Patent Office questions the quantity of APC and its sufficiency, no support for this mere speculation has been provided. Thus the burden of proof has not been shifted to applicants.

The Office Action also asserts that it is unclear "what is the generic 'product' claimed in the instant invention which is detrimental to the activated T cells." (Paper 15, page 4, lines 20-22.) Applicant respectfully traverses. The specification clearly describes detrimental product.

> Detrimental products which have been found to successfully inactivate or ablate activated antigen-specific T cells include CTLA4Ig, a fusion protein which binds to and blocks costimulatory B7 molecules on APC cells, Fas ligand, and antibodies to Fas itself. Antibodies which block costimulatory B7-1 and –2 molecules can also be used.

Page 7, lines 12-15. Thus the specification defines detrimental products as molecules that successfully inactivate or ablate activated antigen-specific T cells. The specification further defines a detrimental product by listing five specific examples. The specification thus adequately teaches one of skill in the art what the detrimental product is that is used in the claimed method.

The Office Action further alleges that the application does not describe when and how the transfected or transduced APCs are administered to a patient with an autoimmune disease. The Office Action asserts, "Applicants did not teach when and how to administer APCs transfected with (1) AchR (AA 1-210), (2) FasL and (3) truncated FADD, to a patient with an auto-immune disease." The specification, however, does disclose how the transfected APCs are administered to a patient. "Transduced or transfected autologous antigen presenting cells are re-introduced to the patient using standard techniques for transfusing blood cells." (Page 7, lines 5-6.) Applicant need not teach what is well known in the art. Clearly one of skill in the art would know how and when to administer APCs, possibly derived from blood or bone marrow, to a patient with an autoimmune disease. For example, Malech et al. teach that "transduced PBSCs derived from the apheresis products were administered intravenously." See also 12135 first column, first full paragraph. (Page 12134, first column, lines 3-4; Tab B) Thus this aspect of the invention is enabled by both the teachings of the specification and the prior art.

The Office Action discounts the evidence which applicant provided in the last amendment to demonstrate enablement, asserting "that the prior art on *ex vivo* therapy referred to by applicants is directed to gene supplementation" (paper 15, page 3, lines 2-3) and that the claimed methods are not *ex vivo* gene therapy drawn to gene supplementation. Rather, the Office Action asserts, the claims are drawn to immunotherapy. Applicant respectfully traverses. Gene supplementation and immunotherapy are not two mutually exclusive therapies. The therapy claimed in the application is *ex vivo* gene therapy. The method claims recite that antigen presenting cells are removed from an autoimmune disease patient. A polynucleotide that

encodes all or a portion of an auto-antigen to which the patient's antigen-specific T cells respond is transferred into the APCs. The APCs are reintroduced into the patient. This is *ex vivo* gene therapy. *Ex vivo* gene therapy involves removal of cells from a patient, transferring genes into the removed cells for expression, and reintroducing the cells into the patient.

Moreover, the *ex vivo* gene therapy recited in the claims is a form of gene supplementation. The gene encoding the auto antigen, which is introduced *ex vivo* into cells by the methods of the instant application, is supplemental to the repertoire of genes normally expressed in those cells. The fact that the encoded protein has a downstream effect on an immune response in the organism does not preclude it from being supplemental.

Gene supplementation during *ex vivo* gene therapy frequently affects an immune response. For example, Bordignon et al. (Science, Vol. 270, 1995, 470-475; Tab A) teach gene therapy to introduce the adenosine deaminase (ADA) gene into the T cells of children with severe combined immunodeficiency (ADA-SCID). The ADA serves to normalize T cell numbers in patients and restore cellular and humoral immune responses. "After 2 years of treatment, long-term survival of T and B lymphocytes, marrow cells, and granulocytes expressing the transferred ADA gene was demonstrated and resulted in normalization of the immune repertoire and restoration of cellular and humoral immunity." (Lines 5-9 of the abstract.) Thus, Bordignon et al. teaches gene supplementation which also constitutes immunotherapy.

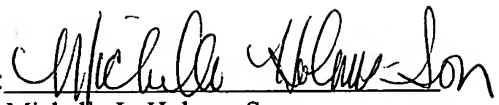
Malech et al. (Proc. Natl. Acad. Sci. USA, Vol. 94, 1997, 12133-12138; Tab B) also teach gene supplementation that serves as a means of providing immunotherapy. Malech et al. teach the introduction of the $p47^{hox}$ gene into $CD34^{+}$ peripheral blood stem cells of chronic

granulomatous disease patients. This gene supplementation affects phagocyte cell number and the ability of patients to fight off infections. "Our data demonstrate the appearance of gene-corrected oxidase-positive granulocytes in the peripheral blood of each of five patients with p47hox CGD after PBSC-targeted gene therapy with vector encoding p47hox. In patient 1, we also demonstrated that the gene-corrected oxidase-positive neutrophils could migrate from the circulation to a site of infection." (Page 12136, column 2, lines 1-6 of the discussion.) Again, Malech et al. demonstrate that gene supplementation can provide immunotherapy. The two categories are not mutually exclusive. Similarly, the immunotherapy of the claimed invention is also an *ex vivo* gene therapy supplementation method. Therefore, the evidence cited in the last amendment involving *ex vivo* supplemental gene therapy is relevant to enablement of applicant's invention.

Applicant has provided disclosure for the method and product claims that would enable one of skill in the art, at the time of the invention, to make and use the invention without recourse to undue experimentation. Withdrawal of this rejection to claims 41-67 is respectfully requested.

Respectfully submitted,

Date: October 15, 2001

By: 
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APPENDIX I. MARKED UP VERSION OF CLAIMS TO SHOW CHANGES MADE.

54. (Amended) Antigen presenting cells of an auto-immune disease patient which are transduced or transfected with a polynucleotide encoding a protein comprising all or a portion of an auto-antigen to which the patient's antigen-specific T cells respond, said all or a portion of an auto-antigen being functionally connected to a signal peptide and a transmembrane/cytoplasmic tail, whereby said all or a portion of auto-antigen is processed by endosomes.

58. (Amended) A virus which infects human APCs and which comprises a polynucleotide which encodes all or a portion of an auto-antigen to which [the] an auto-immune disease patient's antigen-specific T calls responds.

1: Science 1995 Oct 20;270(5235):470-5

Gene therapy in peripheral blood lymphocytes and bone marrow for ADA-immunodeficient patients.

Bordignon C, Notarangelo LD, Nobili N, Ferrari G, Casorati G, Panina P, Mazzolari E, Maggioni D, Rossi C, Servida P, et al.

Telethon Gene Therapy Program for Genetic Diseases, DIBIT, Istituto Scientifico H. S. Raffaele, Milan, Italy.

Adenosine deaminase (ADA) deficiency results in severe combined immunodeficiency, the first genetic disorder treated by gene therapy. Two different retroviral vectors were used to transfer ex vivo the human ADA minigene into bone marrow cells and peripheral blood lymphocytes from two patients undergoing exogenous enzyme replacement therapy. After 2 years of treatment, long-term survival of T and B lymphocytes, marrow cells, and granulocytes expressing the transferred ADA gene was demonstrated and resulted in normalization of the immune repertoire and restoration of cellular and humoral immunity. After discontinuation of treatment, T lymphocytes, derived from transduced peripheral blood lymphocytes, were progressively replaced by marrow-derived T cells in both patients. These results indicate successful gene transfer into long-lasting progenitor cells, producing a functional multilineage progeny.

Publication Types:
Clinical trial

PMID: 7570000 [PubMed - indexed for MEDLINE]

Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 12133–12138, October 1997
Medical Sciences

Prolonged production of NADPH oxidase-corrected granulocytes after gene therapy of chronic granulomatous disease

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Communicated by Roscoe O. Brady, National Institute of Neurological Disorders and Stroke, Bethesda, MD, September 3, 1997 (received for review July 7, 1997)

ABSTRACT **Little is known about the potential for engraftment of autologous hematopoietic stem cells in human adults not subjected to myeloablative conditioning regimens. Five adult patients with the p47^{phox} deficiency form of chronic granulomatous disease received intravenous infusions of autologous CD34$^+$ peripheral blood stem cells (PBSCs) that had been transduced *ex vivo* with a recombinant retrovirus encoding normal p47^{phox}. Although marrow conditioning was not given, functionally corrected granulocytes were detectable in peripheral blood of all five patients. Peak correction occurred 3–6 weeks after infusion and ranged from 0.004 to 0.05% of total peripheral blood granulocytes. Corrected cells were detectable for as long as 6 months after infusion in some individuals. Thus, prolonged engraftment of autologous PBSCs and continued expression of the transduced gene can occur in adults without conditioning. This trial also piloted the use of animal protein-free medium and a blood-bank-compatible closed system of gas-permeable plastic containers for culture and transduction of the PBSCs. These features enhance the safety of PBSCs directed gene therapy.**

Chronic granulomatous disease (CGD) is a rare inherited disorder of phagocytes associated with recurrent life-threatening infections (1, 2). CGD is caused by a defect in the phagocyte NADPH oxidase (phox) that normally generates superoxide. When normal phagocytes engulf opsonized pathogens, the oxidase becomes activated by translocation of three cytoplasmic proteins (p47^{phox}, p67^{phox}, and rac-2) to the cell membrane where they bind to flavocytochrome b_{558} (a heteromeric transmembrane protein composed of two peptides, gp91^{phox} and p22^{phox}) (3, 4). The genetic basis of CGD is heterogeneous (1, 5). The most common form (about two-thirds of the cases) is X chromosome-linked, resulting from mutations in the gp91^{phox} gene. The next most common form (about one-third of the cases) is autosomal recessive resulting from mutations in the p47^{phox} gene on chromosome 7 (2, 4). The remaining 5% of cases are due to mutations in the genes encoding p22^{phox} (chromosome 16) or p67^{phox} (chromosome 1).

Bone marrow transplantation can cure CGD (6, 7), indicating that the stem cells giving rise to granulocytes and monocytes are an appropriate target for gene therapy. Bone marrow transplantation in CGD has been associated with unacceptably high rates of morbidity, mortality, and graft failure, except in the case of HLA-matched sibling donors (6, 7). Specific gene therapy of autologous peripheral blood stem cells (PBSCs) would avoid these problems. The feasibility of genetic correction of CGD with retrovirus vectors has been demonstrated *ex vivo* by transduction of human CD34$^+$ PBSCs from patients with each of the four forms of CGD (8–10). Furthermore, genetic correction of the gp91^{phox} and p47^{phox} deficiency forms of CGD has been demonstrated *in vivo* after stem-cell gene therapy of gene knockout CGD mice and is associated with an increased resistance to infection (11, 12).

In the CGD mouse gene therapy studies, total body radiation was used as a conditioning regimen to enhance engraftment of gene corrected stem cells. Although partial marrow ablation has been thought to be required to optimize engraftment of infused hematopoietic stem cells even in the autologous setting, a number of animal studies using syngeneic cells have suggested that infusion of large numbers of stem cells can partially overcome this barrier (13). In this clinical trial of gene therapy, we examine the potential for engraftment of transduced-gene-corrected autologous CD34$^+$ stem cells in adult patients with the p47^{phox} deficiency form of CGD (p47^{phox} CGD) without marrow conditioning.

MATERIALS AND METHODS

Patients and Consent Documents. Patients 1 to 5 have p47^{phox} CGD as demonstrated by history of recurrent infections, by phagocytic cells that lack both oxidase activity and p47^{phox} protein, and by p47^{phox} gene mutation analysis (14, 15). Patients 1 to 5 are Caucasian and are, respectively, female, male, female, male, and female, and years of age at study entry were 37, 21, 18, 27, and 27. A gene-therapy phase I protocol with associated informed consent document was reviewed and approved by the National Institute of Allergy and Infectious Disease human investigation review board (Protocol 95-I-0134), by the National Institutes of Health Biosafety Committee (Approval document RD-94-XI-05), by the National Institutes of Health Recombinant DNA Advisory Committee (Protocol 9503–104), and by the U.S. Food and Drug Administration (BB IND 6100).

Protocol Clinical Procedures. Beginning on study day 1, patients were given six daily subcutaneous injections with granulocyte colony-stimulating factor (Amgen) at 10 μg/kg to mobilize CD34$^+$ PBSCs from the marrow (16). On both study days 5 and 6, a 10- to 15-liter apheresis stem cell collection was performed by using the CS3000 Plus blood cell separator

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Abbreviations: PBSC, peripheral blood stem cell; CGD, chronic granulomatous disease; phox, phagocyte NADPH oxidase; PMA, phorbol myristate acetate; NBT, nitroblue tetrazolium dye; DHR, dihydrorhodamine 123.

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(Baxter Healthcare, Fenwal Division, Deerfield, IL) with manufacturer recommended settings. On study days 8 and 9, the purified cultured and transduced PBSCs derived from the apheresis products were administered intravenously.

Purification of CD34$^+$ PBSCs. CD34$^+$ PBSC enrichment from the apheresis product was performed by using the ISOLEX 300 SA immunomagnetic stem cell selection system (Baxter Healthcare, Immunotherapy Division, Irvine, CA). The purification procedure using this device was performed by the manufacturer's instructions. Briefly, CD34$^+$ PBSCs were labeled with murine anti-CD34$^+$ mAb and the labeled cells were magnetically captured by using paramagnetic beads containing surface-bound sheep anti-mouse IgG. CD34$^+$ cells were released from the paramagnetic beads by epitope competition by using a peptide that mimics the CD34 epitope as a releasing agent. In some cases, a few additional CD34$^+$ cells could be released from the beads enzymatically by using chymopapain. CD34$^+$ PBSCs were enumerated by fluorescent antibody flow cytometry analysis (16).

Closed Container System for Handling and Culturing CD34$^+$ PBSCs. Purified CD34$^+$ PBSCs were handled, cultured, and transduced in a closed system of plastic containers that could be connected sterilely by using a Terumo SCD 312 sterile tubing welder (Baxter, Fenwal Division). Three types of plastic containers were used. Polyvinyl chloride (PL-146) containers (Baxter, Fenwal Division) with limited gas permeability were used to store medium. CD34$^+$ PBSCs were cultured in gas-permeable stem cell culture (PL-2417) containers (Baxter, Immunotherapy Division) that are optimized for growth of PBSCs. Because of differences in tensile properties and construction, the early prototype PL-2417 containers available for this study were not centrifuged, but instead Life Cell gas-permeable (PL-732) containers (Baxter, Immunotherapy Division) designed for lymphocyte culture were used to centrifuge cells.

Production of Clinical Grade Retrovirus Encoding p47^{phox}. The ORF of human p47^{phox} cDNA (17, 18) was inserted into the MFGS retrovirus vector (Cell Genesys, Foster City, CA) (8–10). MFGS-p47^{phox} plasmid was transfected into the amphotropic envelope packaging line ψ-CRIP, and a vector-producing clone was selected (8–10). For production of cGMP (U.S. Food and Drug Administration current good manufacturing practice) clinical lots of retrovirus supernatant (Cell Genesys), the producer was expanded in DMEM/10% calf serum and then washed with and switched to a serum-free and animal-protein-free medium (X-VIVO 10, BioWhittaker) containing 1% human serum albumin (Baxter Healthcare, Hyland Division, Glendale, CA) for each 8-hr retrovirus supernatant harvest.

PBSC Culture and Transduction Procedures. On the day of apheresis (culture day 0) purified CD34$^+$ PBSCs were suspended at 0.5 to 2 $\times$ 10^6 cells per ml in PBSC growth medium that was serum-free and animal-protein-free (X-VIVO 10 containing 1% human serum albumin and Pixykine [PIXY321; interleukin 3/granulocyte–macrophage colony-stimulating factor fusion protein from Immunex] at 100 ng/ml and granulocyte colony-stimulating factor (Amgen) at 10 ng/ml. PBSCs were cultured overnight in a PL-2417 gas-permeable container in 7% CO_2/93% air at 37°C. The next morning (culture day 1) the cells were transferred to a PL-732 container, centrifuged, and resuspended into 50% vector supernatant (titer ~10^6 transducing units/ml) containing the same growth factor concentrations and at the same cell concentration as for overnight culture. Cells were spin-transduced at 1,200 $\times$ *g* at 32°C for 1 hr (10) and incubated 5 hr at 37°C, 7% CO_2/93% air, after which cells were transferred back to PBSC growth medium in the PL-2417 container overnight. The transduction procedure was repeated on culture days 2 and 3 after which the cells were washed and resuspended in Plasmalyte (Baxter, Hyland Division) containing 1% human serum albumin for intravenous administration. Samples of cells were retained in liquid culture or plated in agarose for further analysis. Cultures of nontransduced PBSCs from the patients and PBSCs from normal volunteers (Human Investigation Review Board approved National Institutes of Health Protocol 94-I-0073) served as negative and positive controls for assays of oxidase activity.

Analysis of Transduction and Correction of Oxidase Activity. At the end of culture day 3, PBSCs were plated in agarose to allow formation of myeloid colonies, which were evaluated for oxidase activity by using a phorbol 12-myristate 13-acetate (PMA)-stimulated nitroblue tetrazolium dye (NBT) test (8, 10). Myeloid colonies demonstrating intense staining with the formazan precipitate were scored as positive. CD34$^+$ cells were maintained in liquid culture for 17 days and analyzed for PMA-stimulated superoxide production by using a chemiluminescence assay (8–10). SDS/PAGE and immunoblotting were used to detect production of p47^{phox} protein in these cells (8, 10).

A flow cytometry assay of oxidant production using dihydrorhodamine 123 (DHR) loading of the cells also was performed (10, 12, 19, 20). At day 17, liquid cultures of normal CD34$^+$ PBSCs undergoing myeloid differentiation contain 10–12% granulocytes, which are the only cells that fluoresce brightly in the flow cytometry DHR assay after PMA stimulation (10). The DHR assay also was used to detect oxidase-positive neutrophils *in vivo* in the peripheral blood of patients after gene therapy (19, 20). Correction of peripheral blood neutrophils also was evaluated visually by NBT staining (21).

Vector copy number in the *ex vivo*-transduced CD34$^+$ PBSCs was determined by Southern blot hybridization (22), and a PCR assay was used for detection of transduced p47^{phox} cDNA present *in vivo* in peripheral blood leukocyte genomic DNA (23, 24). For the PCR assay, nested oligonucleotide primers derived from p47^{phox} cDNA sequence were designed to overlap exon junctions (14, 15) and were found empirically to amplify p47^{phox} cDNA sequence but not genomic sequence. The outer primer pairs are AGCACTAT/GTGTACATGTTCC (bp 65–85, exons 1/2) and GACGTATGGCTCAC/CTGCATAGTTG (bp 696–672, exons 8/7). The inner primer pairs are CTACGAGTTCCAT/AAAACC (bp 140–169, exons 2/3) and CCGGTGATGT/CTGTCGCGG (bp 481–443, exons 5/4). Two detection methods were used. The inner primer pair was used to produce a labeled sequence to probe the outer primer pair PCR product by Southern blotting or was used in a second PCR to amplify a specific nested PCR sequence from the first PCR product (24).

Safety Testing of Patient Blood. Because all five patients have a protein null phenotype of p47^{phox} CGD, patient serum was tested for the development of antibody specific to p47^{phox} by SDS/PAGE and immunoblot (8) detection of recombinant p47^{phox}. Genomic DNA from patient peripheral blood cells was screened for the presence of replication competent retrovirus by using a PCR assay to detect sequence encoding amphotropic envelope (22, 24).

RESULTS

***Ex Vivo* Culture and Transduction of CD34$^+$ PBSCs in Serum-Free Medium and Gas-Permeable Containers.** Patients were free of active infection at study entry, though patient 1 had recovered recently from a pneumonia. Blood studies were within normal limits except for mild anemia (all hematocrits were >26). As expected (16), granulocyte colony-stimulating factor mobilization of CD34$^+$ cells to the peripheral blood of CGD patients was modest and varied among CGD patients. By day 5, the concentration of CD34$^+$ cells had increased from baseline levels below two cells per μl in all patients to a level in patients 1 to 5 of 53, 27, 22, 81, and 13 CD34$^+$ cells per μl, respectively. Despite the apheresis procedure on day 5, these counts were similar at the time of the day 6 apheresis. Ten apheresis products were collected (2 per patient) averaging 35 $\pm$ 3.7 $\times$ 10^9 mononuclear cells per product (mean $\pm$ SEM). After ISOLEX immunoaffinity selection, a mean of 125 $\pm$

23.6×10^6 cells ($n = 10$) was recovered, at a median purity of 80% $CD34^+$ cells resulting in a median yield of 38%.

After the 3 days of culture and transduction, each patient received two autologous products (Table 1, preparations A and B) without any symptoms or changes in vital signs. The total number of cells infused ranged from 0.1 to 4.7×10^6 cells per kg (Table 1). Transduced PBSCs averaged 91 ± 2.7% cell viability, had a colony plating efficiency of 104 ± 38.4 colonies per 1,000 cells plated, and passed safety testing for sterility, endotoxin, and replication competent retrovirus. SDS/PAGE and immunoblotting demonstrated a strong positive signal for the presence of recombinant $p47^{phox}$ protein in all of the transduced CGD PBSC cultures (Fig. 1, lanes B and C). Shown in Table 1 are studies performed on each product to assess the percent of cells expressing CD34 antigen on culture day 3, correction of oxidase activity by chemiluminescence and DHR assays on culture day 17, the percent of myeloid-colony-forming progenitors plated on day 3 giving rise to oxidase-positive colonies, and the vector copy number in the transduced PBSCs. The data are consistent with preservation of a primitive phenotype and a high rate of gene transfer and functional correction *ex vivo*. Though not shown, nontransduced PBSC cultures from the 10 apheresis products from the five patients demonstrated less than 1% of normal chemiluminescence and DHR assay and gave rise to no NBT-positive myeloid colonies. When 17-day-cultured PBSCs from the CGD patients were compared with cultured PBSCs from normal individuals, similar numbers of granulocytes are present by morphological examination in transduced and nontransduced cultures of CGD PBSCs and in the cultures of normal PBSCs, but highly fluorescent oxidase positive cells were detected in the flow cytometry DHR assay only in transduced cell cultures from patients as reported in Table 1 and from normal individuals. Though not shown, the mean fluorescence per corrected patient granulocyte in the transduced cultures was similar to that seen with granulocytes derived from cultured normal PBSCs, suggesting full restoration of oxidase activity in gene-corrected granulocytes *ex vivo*.

Presence of NADPH Oxidase-Positive Neutrophils in the Peripheral Blood After Intravenous Administration of *Ex Vivo* Transduced Autologous $CD34^+$ PBSCs. The flow cytometry DHR assay was used to measure the appearance of NADPH oxidase-positive granulocytes in the peripheral blood after gene therapy. The dot plot shown in Fig. 2*A* demonstrates that the events generated from analysis of PMA-stimulated normal peripheral blood granulocytes cluster in a tight band at the right side of the graph, consistent with robust oxidase activation. As shown in Fig. 2*B*, analysis of PMA-stimulated blood granulocytes from patient 1 before gene therapy resulted in all but a single event to the left of the "positive threshold" line, characteristic of absent oxidase function. At day 24 after gene therapy, PMA-stimulated peripheral blood granulocytes from patient 1 generated almost 80 events appearing to the right of the "positive threshold" line in a tight cluster (Fig. 2*C*) with mean fluorescence intensity (*x* axis) similar to that of granulocytes from the normal control (compare Fig. 2*A* and *C*). Although the number of corrected cells is small, the data indicate that these gene corrected granulocytes from patient 1 have acquired oxidase activity similar to normal cells. Qualitatively similar results were obtained for the other four patients. To confirm the results of the DHR assay by direct visualization of individual neutrophils, an NBT stain of PMA-stimulated peripheral blood neutrophils was performed. In the example shown in Fig. 3 with blood from patient 1 at day 26 after gene therapy, 1 in 2,000 granulocytes was oxidase-positive, consistent with the count determined by DHR assay as reported in Fig. 4.

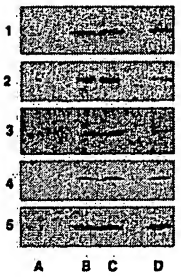

FIG. 1. Correction of $p47^{phox}$ protein deficiency *ex vivo*. These are SDS/PAGE immunoblots demonstrating detection of $p47^{phox}$ protein in transduced or control $CD34^+$ PBSCs at culture day 17. The results for studies of patients 1 to 5 are shown from the top to bottom, as indicated. Shown in lanes A are the analyses of nontransduced cultured $CD34^+$ PBSCs from each patient and as expected no signal is detected. Shown in lanes B and C are the analyses of cultured and MFGS-$p47^{phox}$-transduced $CD34^+$ PBSCs derived from the first and second apheresis products from each patient. Shown in lanes D as a positive control in each case is an analysis of nontransduced normal control $CD34^+$ PBSCs cultured in parallel with the patient cells.

Each patient was followed over time for detection of corrected granulocytes in peripheral blood (Fig. 4). In each subject no oxidase-corrected granulocytes were detected for at least 2 weeks

Table 1. Evaluation of autologous PBSC after *ex vivo* transduction and culture

Patient*	Prep	Cells infused†	% $CD34^+$‡	% of normal chemiluminescence§	DHR assay,§ % granulocytes corrected	% NBT-positive colonies¶	Vector copy number‖
1	A	60	85	25	30	9	0.05
	B	200 (4.7)	97	26	21	6	0.11
2	A	12	84	26	90	29	0.19
	B	45 (0.9)	94	23	44	28	0.08
3	A	215	61	65	75	14	0.13
	B	66 (4.3)	85	64	75	18	0.18
4	A	77	92	27	34	9	0.16
	B	129 (2.5)	81	36	63	11	0.11
5	A	2	63	32	27	19	0.13
	B	2 (0.1)	79	39	59	14	0.13

*Each patient received by vein two preparations (Prep, A and B) of the transduced and cultured PBSCs derived from the first and second apheresis procedures, respectively.

†At culture day 3 for each preparation, the number of cells shown ($\times 10^{-6}$) were infused intravenously. Shown in the parentheses is the total number of cells ($\times 10^{-6}$) (A plus B) infused per kg of body weight.

‡Measured by flow cytometry analysis at the end of culture day 3.

§Assays were performed on day 17 of culture.

¶Cells were plated at culture day 3 and assayed 14 days later.

‖Measured by Southern blot of genomic DNA from the transduced and cultured PBSCs, probed with a MFGS-vector-specific 5′ long terminal repeat sequence and a cell line with known vector copy number of 1 as a reference.

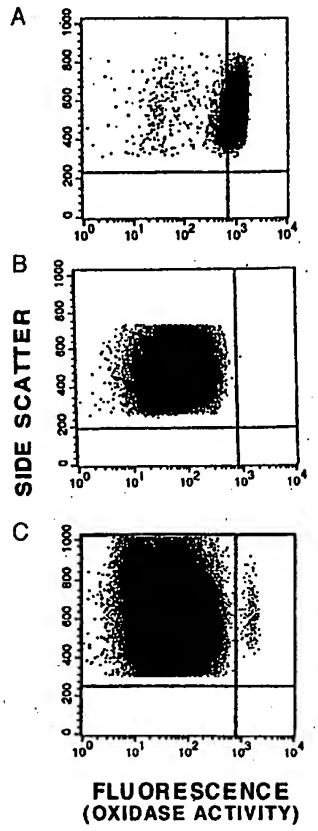

FIG. 2. Correction of neutrophil oxidase activity *in vivo*. These are dot plots of the flow cytometry DHR assays of oxidant production by PMA-stimulated peripheral blood granulocytes. Shown are analyses of granulocytes. (*A*) Normal volunteer. (*B*) Patient 1 before gene therapy. (*C*) Patient 1 at 24 days after gene therapy. Each event (dot) represents the analysis of parameters derived from a single cell. The data shown have been gated to include only events with the forward × side scatter characteristics of granulocytes. Data are plotted to evaluate fluorescence (*x* axis) as a measure of oxidase activity and side scatter (*y* axis) as a means of distinguishing individual granulocytes (events). The "positive threshold" vertical line is set so that 95% of stimulated normal granulocytes are to the right of that line in the region defined as oxidase positive.

after transplantation of the autologous transduced PBSCs. After that time, an increasing number of oxidase-positive granulocytes could be detected in the peripheral blood. The peak response occurred between day 25 (patient 3) and day 53 (patient 2) with a mean of 35 days. The maximum percent of oxidase-positive granulocytes at the peak ranged from 0.004% (patient 2) to 0.051% (patient 1) with a mean of 0.019% (about 1 in 5,000 cells). The range of duration of detection of oxidase-corrected granulocytes was 51 days (patient 5) to 172 days (patient 3) with a mean of 118 days. Of note is that the kinetics of appearance of oxidase-corrected cells did not rise to a single peak followed by a smooth decay over time but instead appeared to rise and fall several times over the duration of detection of positive signal. For example, patient 3 demonstrates five (possibly six) maxima at days 25, possibly 32, 39, 53, 102, and 172.

It is of note that the greatest number of transduced PBSCs were administered to patients 1, 3, and 4 in that rank order and that these same three patients showed the longest duration of detection of corrected granulocytes. Furthermore, patients 1 and 3 had the highest peak number of such corrected granulocytes. Patients 2 and 5, in that rank order, had far fewer transduced PBSC transfused and this correlated with patients 2 and 5, in that order,

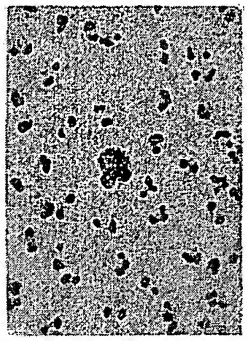

FIG. 3. Correction of neutrophil oxidase activity *in vivo*. This photomicrograph shows NBT-stained PMA-stimulated neutrophils from peripheral blood of patient 1 at day 26 after gene therapy. The cytospin preparation is counterstained red-orange with safranin to visualize segmented neutrophil nuclei. Shown in the center is a single NBT-positive neutrophil that is partly obscured by the dense blue-black precipitate of formazan, a product of NBT reduction by superoxide. This amount of precipitate is evidence of vigorous production of superoxide by this gene-therapy-corrected neutrophil. A visual count of neutrophils on this slide showed that about 1 in 2,000 cells were NBT-positive.

having the shortest duration and patient 2 having the lowest peak number of corrected granulocytes. Patients 1 to 5 have now been followed after gene therapy for 25, 23, 22, 21, and 20 months (as of August 1997), respectively, but no oxidase-positive cells have been seen in any peripheral blood DHR assay after the last data points shown in Fig. 4. PCR detection of the transduced $p47^{phox}$ cDNA was performed on genomic DNA isolated from peripheral blood leukocytes as shown in Table 2 and confirms that a transient low level of gene marking of peripheral blood leukocytes occurred.

Results of Safety Studies and Long-Term Clinical Follow Up. PCR assay of amphotropic envelope sequence failed to detect evidence of replication-competent retrovirus in peripheral blood leukocytes at 1, 3, 6, and 9 months after gene therapy. Similarly, serum samples were negative for anti-$p47^{phox}$ antibodies at 1 and 3 months after gene therapy. All five patients are currently stable without infection. Except for patient 1, the other four patients have had no deep tissue infections during the follow-up period, and hematologic, renal, and liver function tests are normal. Patient 1 had severe *Burkholderia cepacia* pneumonia 3 weeks after gene therapy, from which she recovered. During her pneumonia oxidase-positive neutrophils were detected in an empyema by flow cytometry DHR assay and NBT stain, demonstrating that these gene therapy corrected cells were capable of migrating to an inflammatory focus. It is possible that host responses to this infection affected the peak level of gene corrected granulocytes seen in this individual.

DISCUSSION

Our data demonstrate the appearance of gene-corrected oxidase-positive granulocytes in the peripheral blood of each of five patients with $p47^{phox}$ CGD after PBSC-targeted gene therapy with vector encoding $p47^{phox}$. In patient 1, we also demonstrated that the gene-corrected oxidase-positive neutrophils could migrate from the circulation to a site of infection. Moreover, the kinetics of appearance of these functionally corrected granulocytes share similar characteristics in all patients. In all patients, the first appearance of oxidase-positive granulocytes required at least 2 weeks, suggesting that engraftment, proliferation, and differentiation of the transduced PBSCs in the marrow was required. In all patients, there was an initial wave of oxidase-positive cells first peaking at 22–39 days followed by one or more waves (usually of much smaller magnitude) of oxidase-positive cells at intervals out to 6 months in some individuals. If this periodicity is real, it could be evidence of clonal succession where only a small subset

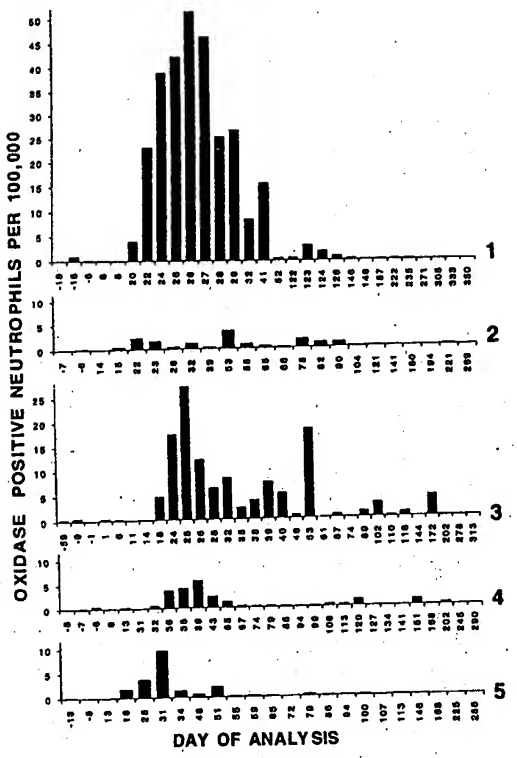

FIG. 4. Prolonged production of oxidase-corrected granulocytes *in vivo*. These bar graphs demonstrate over time the proportion of oxidase-positive neutrophils in the peripheral blood after gene therapy of the five CGD patients. For each data point, flow cytometry DHR assay was performed on peripheral blood leukocytes and the number of oxidase-positive neutrophils was determined as shown in Fig. 2. From top to bottom, the results from analyses of blood from patients 1 to 5, respectively, are shown. For all patients, the vertical axis (oxidase-positive neutrophils per 100,000 cells) is the same scale allowing direct visual comparison between patients. However, the horizonal axis is not proportional and the numbers beneath the data bars indicate the days of analyses relative to the first intravenous administration of transduced autologous $CD34^+$ PBSCs and differ for each patient.

of primitive progenitors in the marrow contribute to active hematopoiesis at any time (25).

Most studies of gene marking of autologous PBSCs have subjected patients to the myeloablative conditioning usually associated with cancer therapy (ref. 26; for review, see ref. 27). Less is known about engraftment potential of autologous progenitors without ablative marrow conditioning. With some disorders such as adenosine deaminase-deficient severe combined immune deficiency or Fanconi syndrome, gene-therapy-corrected lymphocytes or hematopoietic progenitors may have a selective growth advantage over uncorrected cells (28–31). In reports of the use of transduced hematopoietic blood stem cells to treat adenosine deaminase-deficient severe combined immune deficiency without myeloablation, there is evidence of prolonged engraftment (30, 31). The three newborn infants with adenosine deaminase-deficient severe combined immune deficiency treated with transduced autologous cord blood stem cells show evidence of rising levels of gene-marked lymphocytes, though marking of myeloid cells remains very low in a range similar to that seen in our present study (31). Because correction of adenosine deaminase deficiency should provide a selective growth advantage specifically to lymphocytes, this finding might have been expected. In addition, cord blood is a particularly rich source of stem cells and infants and very young children may be more receptive to engraftment of autologous cells. It remains to be seen whether

Table 2. PCR detection of gene marking

Pt 1		Pt 2		Pt 3		Pt 4		Pt 5	
Day*	PCR†	Day	PCR	Day	PCR	Day	PCR	Day	PCR
−6	−	−3	−	−8	−	−6	−	−15	−
28	+	24	+/−‡	3	−	29	+	−3	−
33	+	28	−	26	+	33	+	29	−
124	−	68	−	55	+	40	+	34	+
147	−	93	−	70	+	65	−	61	−
241	−	190	−	92	−	89	−	82	−
313	−	357	−	119	−	118	−	117	+
362	−	147	+	132	−	139	−		
		175	−	352	−	335	−		
		205	−						
		232	−						

Pt, patient.
*Number of days before or after gene therapy.
†Vector $p47^{phox}$ cDNA sequence detected (+) or not detected (−) in blood leukocytes. All + were <0.1% of cells marked.
‡The +/− indicates positive signal detected by Southern blotting of the PCR product but not by nested primer detection.

permanent high-level engraftment of autologous-gene-marked hematopoietic stem cells can be achieved in nonconditioned older children or adults, where gene transfer provides no selective growth advantage.

We have demonstrated in a congenic mouse model system that low-dose nonablative radiation conditioning can increase greatly the engraftment of congenic marrow stem cells in a radiation dose-dependent manner (32). This suggests the possibility that acceptable regimens of marrow conditioning may be developed for hematopoietic-stem-cell-targeted gene therapy. Such conditioning might increase the level of gene marking from that seen in our current study to levels that could provide prolonged clinical benefit.

The DHR assay of oxidase function provides strong evidence that low-level prolonged engraftment of gene-marked hematopoietic progenitors can occur in human adults without marrow ablation or conditioning. Though the PCR confirmed this, PCR was not sensitive enough to detect marking at later time points where the flow cytometry DHR assay continued to indicate lower levels of oxidase-positive neutrophils. The PCR data demonstrate that the eventual disappearance of gene-corrected oxidase-positive granulocytes by DHR assays was not associated with continued presence in the peripheral blood of substantial numbers of leukocytes marked with a nonfunctional transduced gene. Although we cannot exclude the possibility that silencing of transcription of the transduced oxidase gene is occurring, the data are more consistent with disappearance of transduced cells. This might happen if very early progenitors rather than permanently repopulating stem cells were targeted. Several published human clinical studies of hematopoietic-stem-cell-targeted gene transfer (26, 27, 30, 31) have demonstrated low-level engraftment of retrovirus-transduced gene in blood or marrow cells by using PCR. Although these studies indicate the presence of the transduced gene, assessment of gene function in the host, as in our study and others (33), provides an important additional insight regarding the clinical potential for gene therapy. How to target the most primitive stem cells and how to prevent transcription silencing *in vivo* remain important issues to resolve in future studies.

An additional goal of this clinical trial was to develop and pilot the use of materials and methods that increase the safety of *ex vivo* gene therapy targeting hematopoietic stem cells. Animal proteins, including fetal calf serum, are widely used as required supplements to most cell culture media. Animal proteins internalized by human cells during prolonged culture may not be removed by centrifugation washing and can stimulate an immune response (34). Because gene therapy is in an early developmental stage, it is likely that any patients participating in these initial

studies will be treated again in the future at a time when such treatments are more efficient. If it is at all possible to limit exposure to animal proteins, particularly fetal calf serum, in these early studies without compromising the scientific goals of the study, then such a safety feature should be incorporated into the protocol. A second important safety feature incorporated into this study was a closed system of gas-permeable flexible plastic containers for culture and transduction. The closed system reduces the contamination risk associated with pipetting cells and medium yet allows such handling to become a counter-top process. Biosafety cabinets are required at only a few steps and the system is compatible with techniques already used widely in most blood banks. We demonstrate that it is possible to incorporate these safety features without compromising PBSC viability or transduction efficiency.

The clinical potential of gene therapy is yet to be realized, and there has been considerable interest in defining both the scientific and clinical goals of human trials of gene transfer. In the case of CGD, where life-threatening infections may require many weeks or months of therapy and relapses are frequent, use of gene therapy to provide even short- to medium-term production of oxidase-positive autologous granulocytes may be clinically beneficial. This concept is supported by published studies of gene therapy in mouse models of both the X chromosome-linked ($gp91^{phox}$-deficiency) and $p47^{phox}$-deficiency forms of CGD that demonstrate that even transient partial correction of the oxidase defect is associated with some protection against infection challenge (11, 12). Furthermore, in human female carriers of the X chromosome-linked form of CGD, the X chromosome inactivation that occurs during embryogenesis results in phenotypic mosaicism at the cellular level in which both oxidase-positive and oxidase-negative granulocytes can be detected in the peripheral blood (21). Because this is a stochastic process, some female carriers can be found who have only 3% to 5% oxidase-positive neutrophils yet do not suffer from an increased incidence of infection. The knockout mouse studies and the clinical observations of X chromosome-linked CGD carriers suggest that even a short-term low level of gene correction in CGD could be clinically beneficial for treatment of severe prolonged infections. Until the tools are developed to achieve high-level permanent gene transfer to hematopoietic cells, our studies suggest that an achievable intermediate goal of development of gene therapy for CGD might be to augment neutrophil function in the treatment of severe infections.

Somatix Therapy Corporation, an industrial collaborator during the conduct of this study, is now a part of Cell Genesys. We thank Immunex for providing Pixykine for *ex vivo* culture of $CD34^+$ PBSCs. We are grateful for the important contributions of the National Institute of Allergy and Infectious Diseases 11 East day hospital staff and the National Institutes of Health transfusion medicine apheresis staff. We thank Dr. Stephen Chanock for doing the $p47^{phox}$ mutation analysis of our patients. We thank Dr. Philip Murphy for critical reading of the manuscript and Dr. Douglas Kuhns for preparing Fig. 3. Finally, we thank the participating patients and the physicians who served as physician-advocates for their patients during the informed consent process.

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RAPD COMMUNICATION

Functional Correction of Fanconi Anemia Group A Hematopoietic Cells by Retroviral Gene Transfer

By Kai-Ling Fu, Jerome R. Lo Ten Foe, Hans Joenje, Kathleen W. Rao, Johnson M. Liu, and Christopher E. Walsh

Fanconi anemia (FA) is an autosomal recessive genetic disorder characterized by a variety of physical anomalies, bone marrow failure, and an increased risk for malignancy. FA cells exhibit chromosomal instability and are hypersensitive to DNA cross-linking agents such as mitomycin C (MMC). FA is a clinically heterogeneous disorder and can be functionally divided into at least five different complementation groups (A-E). We previously described the use of a retroviral vector expressing the *FAC* cDNA in the complementation of mutant hematopoietic cells from FA-C patients. This vector is currently being tested in a clinical trial of ex vivo hematopoietic progenitor cell transduction. The FA-A group accounts for over 65% of all FA cases, and the *FAA* cDNA was recently identified by both expression and positional cloning techniques. We report here the transduction and phenotypic correction of lymphoblastoid cell lines from four unrelated FA-A patients, using two amphotropic *FAA* retroviral vectors. Expression of the *FAA* transgene was adequate to normalize cell growth, cell-cycle kinetics, and chromosomal breakage in the presence of MMC. We then analyzed the effect of retroviral vector transduction on hematopoietic progenitor cell growth. After *FAA* transduction of mutant progenitor cells, either colony number or colony size increased in the presence of MMC. In addition, FAA but not FAC retroviral transduction markedly improved colony growth of progenitor cells derived from an unclassified FA patient. *FAA* retroviral vectors should be useful for both complementation studies and clinical trials of gene transduction.

FANCONI ANEMIA (FA) is an autosomal recessive disorder clinically manifested by bone marrow (BM) failure, diverse developmental anomalies, and cancer susceptibility. Although FA patients are phenotypically heterogeneous, the hematologic abnormalities are the most clinically significant.[1] The hallmark of the disease is the hypersensitivity of FA cells to DNA bifunctional cross-linking agents such as mitomycin C (MMC) and diepoxybutane (DEB). FA cells exposed to these clastogenic agents in vitro reproducibly develop chromosomal aberrations and die by apoptosis.

Somatic cell hybridization studies have established at least five complementation subtypes (A-E). The FA-A group is the most prevalent, comprising 60% to 70% of FA cases studied.[3] The identification of the *FAA* cDNA was recently described using a positional and an expression cloning strategy[5] previously used to identify the *FAC* cDNA. *FAA* is located on chromosome 16q24.3 and encodes a predicted protein of 163 kD. The predicted FAA and FAC proteins have no homologies to each other or to other known proteins, suggesting a novel cellular pathway for maintenance of chromosomal stability.

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Submitted March 19, 1997; accepted August 14, 1997.

Supported in part through the Leukemia Society Translational Research Grant No. 6229-96. C.E.W. is a recipient of the Lucille B. Markey Charitable Trust.

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0006-4971/97/9009-0060$3.00/0

Cell fusion studies can reliably assign FA patients to complementation groups, because patients functionally classified as FA-C turned out to have pathogenic mutations in *FAC*. Mutation screening of *FAC* has shown at least 8 pathogenic mutations, IVS4 + 4 A to T and 322delG being the most prevalent.[7,8] Four mutations have so far been reported in *FAA* and work is in progress in several laboratories to determine the mutation spectrum in FA-A patients.

Therapy for FA is directed at the hematologic manifestations, typically the most life-threatening complications. BM aplasia is usually treated with androgens and supportive care. Allogeneic BM transplantation (BMT) from a histocompatible sibling donor is the treatment of choice for patients with severe disease who have a suitable donor. FA patients who undergo HLA-identical sibling BMT have a 2-year survival probability of 66%, but only 29% after alternative donor transplant.[9] Although BMT is potentially curative of the hematologic pathology, patients may go on to develop secondary malignancies, often solid tumors of the head and neck. Umbilical cord blood transplantation has also been successfully applied for FA patients.[10] For FA patients lacking a suitable stem cell donor, other approaches to treatment are needed.

Gene augmentation therapy entails the insertion of a normal gene into an appropriate target cell to correct the function of a defective gene. Viral vectors are currently the primary vehicles due to their efficiency in entering and transferring genetic material into cells. Using two different viral vectors, we previously described the genetic complementation of FA-C lymphoblasts.[11,12] Recombinant virus transduction of the *FAC* cDNA into FA-C CD34$^+$ hematopoietic progenitor cells improved their survival and clonogenic growth. This in vitro survival advantage of corrected FA-C cells suggested that FAC-transduced hematopoietic progenitor cells might have a selective advantage in vivo and led to the first FA clinical gene therapy trial.[13] In this report, we describe the development of two retroviral vectors for the complementation of group A FA hematopoietic cells.

MATERIALS AND METHODS

Viruses and cells. All lymphoblast cell lines were grown in RPMI containing 15% heat-inactivated fetal calf serum and gluta-

mine as previously described.[12] PA317[14] and GP+ E86[15] cell lines were grown as previously described.

FA-A patients and cell lines. Patients 1 (43-year-old woman), and 2 (6-year-old girl) were assigned to complementation group A by somatic cell hybridization and/or mutational analysis (H. Joenje, A. Auerbach, unpublished observations). Patient 3 (8-year-old girl) was previously unclassified by FA complementation group. The following lymphoblastoid cell lines were used, characterized as FA-A by cell fusion experiments: HSC72, which is the original reference group A patient reported,[6] VU5,[3] EUFA471,[5] and EUFA528 (unpublished observations). All patients were diagnosed as having FA on the basis of clinical symptoms and a positive chromosomal breakage test. Confirmation of the group assignment by mutation screening is currently in progress and will be reported elsewhere.

Generation of amphotropic producer clone. The 5.5-kb *FAA* cDNA was subcloned into the *Not* I site of the pG1 retroviral vector to create pG1-FAA5.5. The pG1 vector is based on the Moloney murine retrovirus and does not contain eukaryotic selection markers. The *FAA* cDNA is driven by the Moloney murine retrovirus long terminal repeat (LTR) promoter. The *FAA* termination and polyadenylation signals are maintained in this construct. A truncated 4.3-kb *FAA* cDNA version (pG1-FAA4.3), lacking the 3′ untranslated region, was generated using the Transformer Site-Directed Mutagenesis Kit (Clontech, Palo Alto, CA) and a mutagenic primer (5′-TG-GCAGAGCTG CTGGCTGAAAGCTAGCTAGCGGCCGCTCGA-GGC 3′) according to the manufacturer's protocol. This produces a truncation at nt 4302 leading to a loss of 94 bp in the *FAA* open reading frame (ORF). The vector/insert junctions were verified by sequencing (Sequenase; US Biochemicals, Cleveland, OH). The termination and polyadenylation signals reside in the LTR.

The pG1-FAA4.3 plasmid was cotransfected with a plasmid carrying the neomycin resistance (Neo) gene by calcium phosphate coprecipitation into the PA317 amphotropic packaging cell line. Two days later, the cells were trypsinized, replated, and placed under G418 (Geneticin; GIBCO/BRL, Gaithersburg, MD) selection (500 μg/mL, active). Individual resistant colonies were isolated and analyzed for the presence of viral RNA by dot-blot hybridization. High-titer producer clones were expanded and genomic DNA isolated. Alternatively, the pG1-FAA4.3 plasmid was transfected into the GP + E86 ecotropic packaging cell line grown in media containing 5 mmol/L sodium butyrate. After 2 days, the cell supernatant was removed, filtered, admixed with polybrene (4 μg/mL), and incubated with PA317 cells. Sequential supernatant infection was repeated twice before PA317 cells were diluted and individual colonies obtained. Producer clone 6.9 was identified as a high-titer unrearranged producer clone after dot-blot analysis for viral RNA and Southern blot analysis of the proviral genome. Genomic DNA was digested with *Nhe* I and Southern blot hybridization performed using ammonium acetate buffer[7] and nylon transfer (Hybond N; Amersham, Arlington Heights, IL). The blot was probed with a *Bam*HI fragment P^{32} random primer-labeled fragment of the *FAA* cDNA.

The plasmid pG1FAA-5.5 was cotransfected into GP E86 cells with the Neo plasmid by calcium-phosphate coprecipitation. Neomycin-resistant colonies grown in G418 (500 μg/mL, active) were screened for viral RNA production. Producer clones were identified by viral RNA dot-blot analysis; Southern blot analyses confirmed unrearranged proviral genomes in these cell lines. High-titer ecotropic producer lines 1.16 and 2.16 were identified. Supernatant from these cell lines was used to infect PA317 cells twice daily for three days. Individual colonies were established by limiting dilution and analyzed for the presence of viral RNA and intact proviral genome. Two high-titer producer clones 17 and 29 were identified and used in our studies.

Viral titer of the producer lines was assessed by semiquantitative Southern blot analysis (unpublished results, method provided by Dr E. Vanin, St Jude Children's Research Hospital, Memphis, TN). 5×10^5 NIH 3T3 cells were incubated with various amounts of retroviral supernatant and allowed to incubate overnight. Genomic DNA was isolated and 10 μg DNA digested with *Nhe* I. A 300-bp *Not* I/*Pst* I probe (gift from Dr E. Vanin) from the pG1 backbone was used for hybridization. Supernatant from the previously defined FA-C complementing retroviral producer cell line no. 52.16b, a functional titer of 5×10^6 neomycin-resistant colonies/mL, was used as positive control. BOSC and *FAA* retroviral vectors were generated from the same pG1 vector backbone to which the probe hybridizes. Extended marker rescue assay for detection of helper virus was performed as previously described.[9]

Retroviral gene transfer of Epstein-Barr–transformed lymphoblasts. Lymphoblasts (1×10^5 cells/mL) were incubated with an equal volume of viral supernatant containing protamine sulfate (5 μg/mL). Cells were grown in RPMI supplemented with 15% fetal calf serum for 2 days before 10 nmol/L mitomycin C (MMC) (Calbiochem, La Jolla, CA) was added. Cells were maintained in media containing 10 nmol/L MMC. Non–virus-infected lymphoblasts yielded no viable cells after 5 weeks following drug selection.

MMC sensitivity assay. Cell sensitivity to MMC was assayed as previously described.[12] Lymphoblasts were plated at 1×10^5 cells/mL in 24-well plates. Increasing concentrations of MMC were added and after 4 days, cellular viability assayed by trypan blue exclusion. Samples were performed in duplicate.

Flow cytometry. Lymphoblasts were incubated at 2×10^5 cells/mL with 100 nmol/L MMC for 48 hours or 10 nmol/L MMC for 5 to 7 days. Cell nuclei were stained with propidium iodine using CycleTest (Becton Dickinson, Franklin Lakes, NJ) as described by the manufacturer. Stained cell nuclei were analyzed by FACSCAN (Becton Dickinson, Franklin Lakes, NJ) using Cytomation Cicero Software (Fort Collins, CO). Cell-cycle analysis was performed using MODFIT LX 2.0 (Verity Software House, Topsham, ME).

Cytogenetic breakage. Lymphoblast cultures were analyzed for cytogenetic breakage and exchange figures (radial formation) by exposure to MMC (40 ng/mL final) for 2 days in the dark. Cultures were obtained after a 90-minute exposure to 0.1 μg/mL colcemid and 10 μg/mL ethidium bromide. After a 10-minute treatment with 0.075 mol/L KCl, the cells were fixed with a 3:1 mixture of methanol:acetic acid. Slides were prepared using wet slides, air dried, and stained with Wright's stain. Fifteen or 50 metaphase figures from each culture were scored for obvious breaks, gaps larger than a chromatid width, and exchange figures.

Transduction of CD34 progenitors. BM samples were obtained after receiving informed consent on a protocol approved by the Institutional Review Board of the University of North Carolina Medical School. Somatic cell hybridization studies indicated that patient 1 belonged to the FA-A complementation group (H. Joenje, unpublished). Genomic analysis of DNA from patient 2 showed compound heterozygous deletions in the *FAA* coding sequence (A. Auerbach, unpublished). Patient 3 had not previously been assigned to a FA complementation group. Mononuclear cells were isolated from the marrow by lymphocyte separation medium (Organon Teknika, Durham, NC) and CD34-enriched cells immunoselected using a CellPro column (Bothell, WA) as previously described.[16] $CD34^+$ cells were incubated overnight in Dulbecco's modified essential media (DMEM) containing 10% fetal calf serum, 100 ng/mL stem cell factor (SCF; Amgen, Thousand Oaks, CA), 25 ng/mL interleukin-3 (IL-3; Sandoz, East Hanover, NJ), and 50 ng/mL interleukin-6 (IL-6; Sandoz). Cells were then incubated with retroviral supernatant from clone 17 and 4 μg/mL protamine. Incubation were repeated twice daily for 2 days. Cells were plated at 20,000/plate and then analyzed for colony formation as previously described.[12] In separate experiments using CD34 cells from patient 3, both FAA and FAC retroviral transduction studies were performed. CD34-immunose-

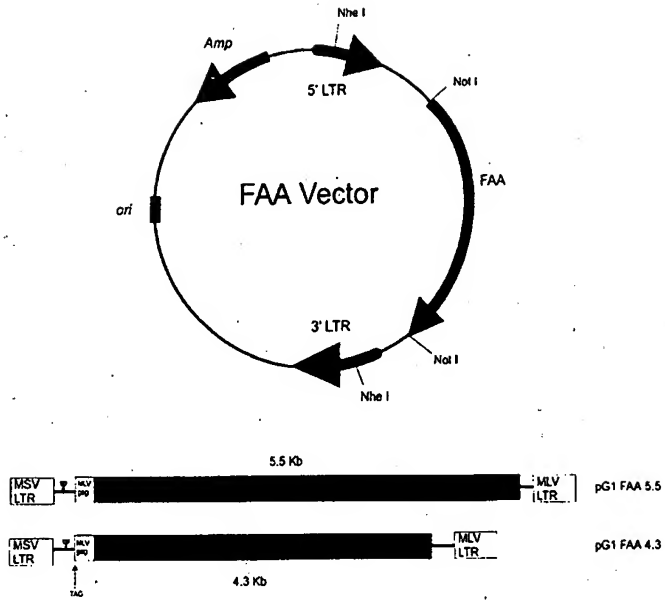

Fig 1. Schematic diagrams of FAA retroviral vector constructs. A general schema for the two retroviral constructs used is shown with the FAA coding region subcloned as a *Not* I fragment into plasmid pG1 (top). (Bottom) The *FAA* cDNA is driven by the 5' hybrid long terminal repeat (LTR) of murine moloney sarcoma virus; psi (ψ) retroviral packaging signal; TAG represents a mutation in authentic start codon in the molony murine leukemia virus *gag*.

lected cells isolated from the umbilical cord blood of a normal newborn were used as control.

RESULTS

Generation of FAA amphotropic producer clones. The *FAA* vector plasmids are shown in Fig 1. pG1FAA5.5 was derived by inserting the 5.5-kb cDNA, isolated from expression cloning, into the plasmid pG1.[10] This cDNA contains an open reading frame of 4,368 bp, a 31-bp 5' untranslated region (UTR) and a 1.1-kb 3'UTR. The sequence upstream of the first ATG has poor homology to a Kozak consensus sequence, and a polyadenylation signal was not found. The plasmid pG1FAA4.3 contains a truncated cDNA lacking the 3' region. This truncated FAA vector was generated to determine if the 3' UTR affected FAA retroviral expression. Amphotropic producer clones for each vector were derived from PA317 cell lines. Genomic DNA subjected to restriction enzyme digestion with *Nhe* I, which cuts once within each LTR, was analyzed by Southern blot hybridization (data not shown). The predicted 6.3- and 5.3-kb fragments were detected, indicating unrearranged proviruses from FAA5.5 clone 17 and FAA4.3 clone 6.9. Proviral copy number was determined by comparisons with dilutions of plasmid pG1FAA estimated to give 0.5 to 2 copies/cell. Southern blot analysis (data not shown) indicated that producer clones 17 and 6.9 had 10 and 3 copy number equivalents, respectively. Titers of each clone were determined by comparison with a producer clone of known copy number (*FLAC* producer clone 52:19).[12] Viral supernatants were incubated overnight on NIH 3T3 cells, genomic DNA isolated and digested with *Nhe* I, and Southern blot hybridization performed. The probe used hybridizes to all pG1-based vectors. The titers of FAA 5.5/17, 5.5/29, and FAA4.3/6.9 were estimated at 5×10^6, 3×10^6, and 1×10^6, respectively, particles/mL (data not shown). Supernatant from each clone was analyzed for replication-competent retrovirus using an extended rescue assay.[19] No replication-competent retroviruses (RCR) was detected from any of the producer clones (data not shown).

Retroviral-mediated gene transfer and phenotypic correction of FA-A lymphoblasts. Epstein-Barr virus immortalized lymphoblast lines established from FA patients were confirmed to be FA-A subtype by somatic cell hybrid complementation and mutational analyses. After viral supernatant infections, each cell line was incubated with low doses of (10 nmol/L) MMC for at least 4 to 6 weeks to select for complemented cells. MMC resistance was then measured by incubating cells at various concentrations of MMC to determine drug sensitivity. All the mutant (parental) lines were markedly sensitive to MMC, with an effective drug concentration yielding 50% reduction in cell viability (IC_{50}) of 2 to 5 nmol/L MMC (Fig 2). However, in marked contrast, retroviral-transduced lymphoblasts were significantly less sensitive to MMC and equivalent in resistance to lymphoblasts established from normal donors. The IC_{50} of both normal and corrected lymphoblasts was greater than 100 nmol/L. Correction to normal was observed in all cell lines tested and with both retroviral constructs. Confirmation of integrated proviruses in *FAA* gene-corrected cells was performed by Southern hybridization of genomic DNA digested with *Nhe* I (Fig 3).

Restoration of cell-cycle progression. Cell-cycle arrest and loss of cellular proliferative capacity are the hallmarks of FA. FA cells typically respond to DNA damage induced by MMC with a delay in the G2 phase of the cell cycle. We tested *FAA* gene-corrected cells to determine if complementation resulted in relief of the G2 checkpoint delay. FA-A cells in each phase of the cell cycle were analyzed by propidium iodide staining and flow cytometry (Fig 4). The percent-

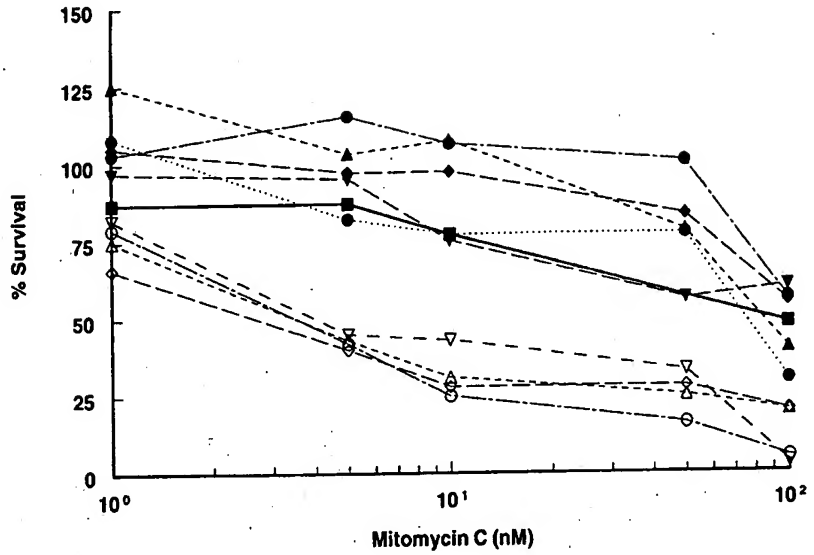

Fig 2. MMC sensitivity of FAA-retroviral transduced lymphoblasts. Plot of cell survival of FAA parental and retroviral-transduced cell lines after incubation with varying concentrations of MMC. MMC sensitivity of lymphoblasts derived from a normal individual is shown. All data points are the results of quadruplicate determinations (SEM < 5%). Open symbols represent FAA mutant parental cell lines, closed symbols represent retroviral-transduced cell lines, (···●···) indicates retroviral-transduced cell line with vector FAA/4.3 (clone 6.9). Normal lymphoblasts are represented by the solid line (——). (—○—), HSC 72; (– –△– –), EUFA 528; (—●—), HSC 72/Retro17; (···●···), HSC 72/Retro6.9; (– –▲– –), EUFA 528/Retro29; (—■—), normal; (– –▽– –), EUFA 471; (—◇—), EUFA 005; (—⊟—), EUFA 471/Retro17; (—▼—), EUFA 005/Retro17.

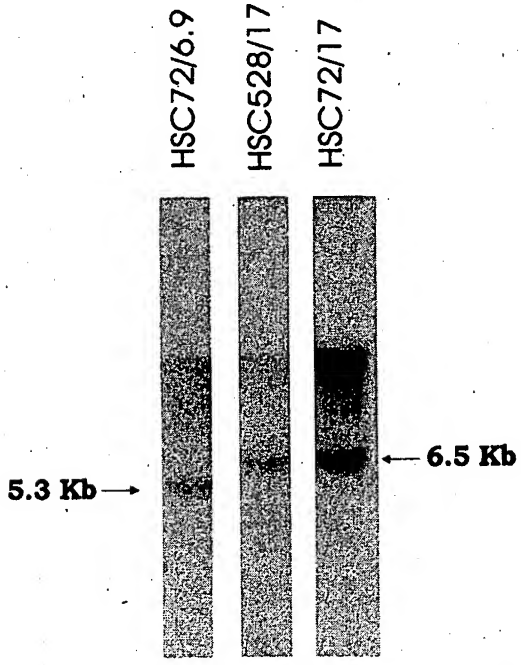

Fig 3. Southern blot analysis of FAA-transduced lymphoblast cell lines. Genomic DNA was isolated from two cell lines transduced with either vector FAA 5.5 or FAA 4.3, digested with *Nhe* I and hybridized with probe to FAA. The expected bands (6.5 kb and 5.3 kb) represent unrearranged proviral form in each cell line.

age of FA-A cells in the G2 phase was increased when compared with normal individuals. Incubation of uncorrected mutant FA-A cells with either low-dose MMC (10 nmol/L) for 1 week or 100 nmol/L for 2 days had the expected effect of increasing the percentage of cells in the G2 phase (Fig 4). Table 1 summarizes the cell-cycle results from five separate experiments. Three different mutant FAA lymphoblast cell lines and three different normal lymphoblasts were studied. Statistical analysis of the percentage of cells in the G2/M cell fraction showed a significant difference ($.02 > P > .01$, Student's *t*-test) when mutant FAA lymphoblasts (35%) were compared with either normal (14.8%) or virus-transduced cells (18.6%) in the presence of MMC. The corrected FA-A cells showed normalized cell-cycle kinetics in both the presence and absence of MMC (Table 1). No significant change in the number of cells in G1/S was observed following the addition of MMC.

Chromosomal breakage analysis of gene-corrected FAA cells. Exposure of all FA cell types to DNA cross-linking agents such as MMC results in a characteristic increased frequency of chromosomal breakage and aberrations. Retroviral-transduced FA-A lymphoblasts were analyzed for chromosomal breakage following incubation with MMC. As shown in Table 2 and Fig 5, the mutant HSC 72 and EUFA 528 lines have a spontaneous breakage rate comparable with normal cells. After drug exposure, the percentage of mutant cells with chromosomal breaks increased dramatically as expected. Complementation with the *FAA* cDNA led to decreased chromosomal breakage rates for both lines tested.

Transduction of primary hematopoietic cells. BM hematopoietic progenitor cells from three FA patients were as-

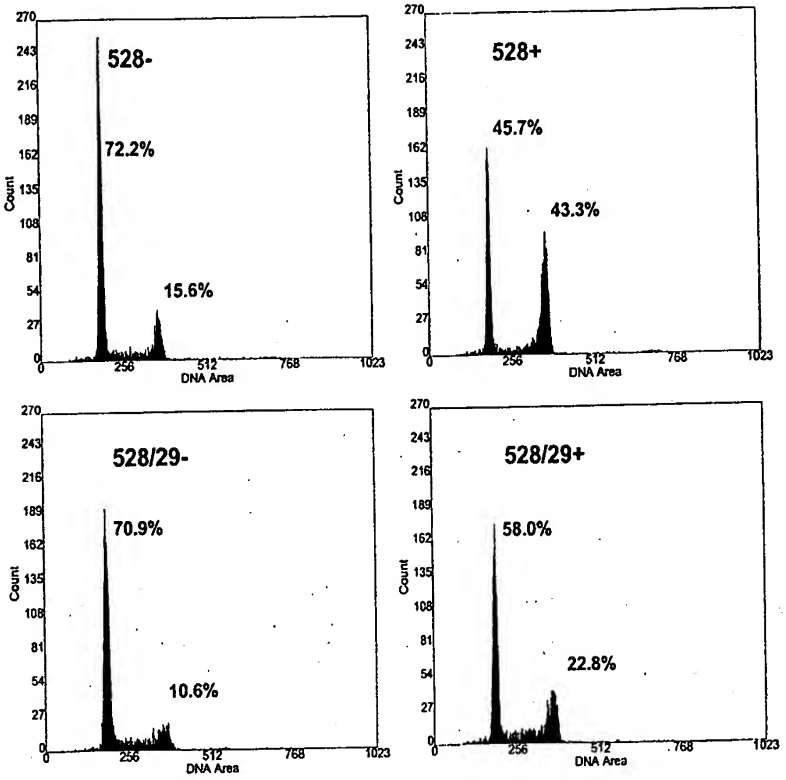

Fig 4. Cell-cycle kinetics of corrected FAA cells. The effect of mitomycin C on DNA flow cytometry histograms of retroviral-transduced lymphoblasts. The transduced and parental FAA cell line EUFA 528 were incubated in the presence and absence of MMC. Cells were harvested, stained with propidium iodide, and DNA histograms obtained 48 hours after cells were exposed to 100 nmol/L MMC. EUFA 528, parental FAA line; EUFA 528/29, retroviral-transduced cell line. −/+ refers to the presence and absence of MMC. The percentage of cells in the G1 and G2/M fractions are indicated.

sayed for colony growth after transduction with our *FAA* retroviral vector. All three patients were pancytopenic and had not received any treatment before donating BM cells. Recent BM morphology showed hypoplasia without blasts and normal karyotypes. BM aspiration, mononuclear cell collection, and $CD34^+$ cell enrichment were performed. Transduction was done over a 3-day period with resuspension in fresh media and viral supernatant each day. After infection, cells were plated in methylcellulose in both the absence and presence of MMC. Colony growth increased slightly following *FAA* virus transduction of cells from patient 1 (see Table 3A). More dramatic was the morphologic difference in the size and type of colonies present following transduction. Mock-transduced colonies were small and granulocytic in appearance, whereas transduced colonies were more robust (larger) and BFU-E, CFU-GM, and CFU-GEMM were observed. As expected, when mock-transduced cells were incubated with low concentrations of MMC, the colony numbers decreased. In contrast, transduced colonies

Table 1. Cell-Cycle Analysis

		% Cells (mean ± SD)*		
Cell Type	Condition	G1/G0	S	G2/M
FAA	−MMC	69.9 (4.0)	17.7 (6.3)	12.2 (2.8)
	+MMC	52.9 (13.0)	11.7 (6.4)	35.0 (7.8)
FAA/Retro	−MMC	65.8 (5.3)	22.1 (6.2)	12.0 (3.4)
	+MMC	60.0 (10.8)	20.5 (7.9)	18.6 (3.8)†
Normal	−MMC	63.0 (3.6)	27.9 (4.5)	8.8 (0.7)
	+MMC	59.0 (11.4)	26.2 (9.1)	14.8 (3.9)†

* Results of 5 separate experiments. Three different FAA mutant cell lines and 3 different normal lymphoblast cell lines were used.

† Significant at $.02 > P > .01$ using Student's *t*-test.

Table 2. Cytogenetic Analysis of FAA Lymphoblast Cell Lines

Cell Line	Condition*	Radials†‡	Breakage§
HSC 72	−MMC	0/15	3/15
HSC 72	+MMC	7/15	15/15
HSC 72/Retro	−MMC	0/50	0/50
HSC 72/Retro	+MMC	4/50	15/50
EUFA 528	−MMC	0/45	1/45
EUFA 528	+MMC	23/45	39/45
EUFA 528/Retro	−MMC	0/45	2/45
EUFA 528/Retro	+MMC	0/45	2/45
Lymphoblasts-NML‖	−MMC	0/50	0/50
Lymphoblasts-NML	+MMC	0/50	0/50

* Cells were incubated in the absence or presence of 40 ng/mL MMC for 36 hours.

† The number of exchange figures (radials)/number of metaphases analyzed.

‡ Greater than 20% radial formation is diagnostic for FA.

§ Number of chromosome/chromatid gaps, fragments, and radials/number of metaphases analyzed.

‖ Normal lymphoblasts.

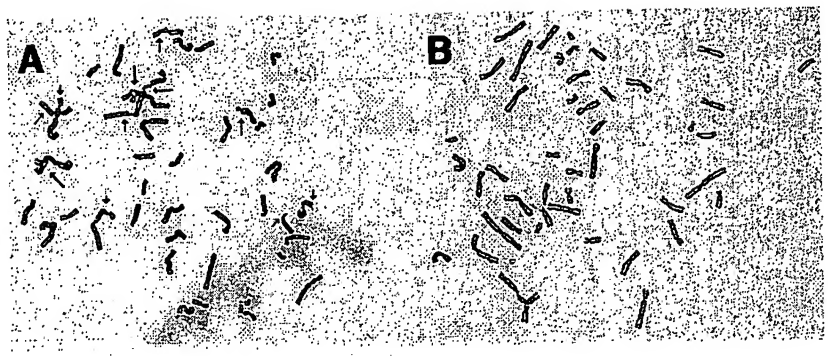

Fig 5. Cytogentic analysis of retroviral-transduced FAA lymphoblasts. (A) A representative metaphase spread from the cell line EUFA 528 following incubation with MMC (40 ng/mL). Chromatid breaks (→), and exchanges (⇒) are indicated. (B) Metaphase preparation of retroviral-transduced EUFA 528 cells following exposure to MMC.

(that were significantly larger in size) grew in the presence of low concentrations of MMC.

Due to limited number of $CD34^+$ cells obtained from patient 2, cells were transduced but not incubated with MMC (Table 3B). In the absence of virus transduction no progenitor colonies were observed. In marked contrast, a significant increase in colony number was observed following virus transduction.

Transduction of $CD34^+$ progenitor cells from an untyped (unknown complementation group) FA patient (no. 3) was also performed (Table 3C) using both FAA and the FAC retroviral infection. Baseline colony growth was inhibited in the presence of 1.0 nmol/L MMC. In the absence of MMC, colony growth increased after incubation of cells with FAA but not the FAC retroviral supernatant. More impressively, in the presence of MMC, colonies resistant to MMC grew only after FAA transduction. Results of colony growth using CD34 cells from umbilical cord blood from a normal newborn are shown for comparison (Table 3D).

Finally, CD34-enriched progenitor cells from a known FA-C patient were transduced with the *FAA* retroviral vector and plated in colony culture. No growth was observed either with or without MMC (data not shown).

Table 3. Colony Growth After Retroviral FAA Transduction of CD34 Cells

	Colony No.*		
Condition (virus/MMC)	BFU-E	CFU-GM	CFU-GEMM
A. Patient 1			
−Virus/−MMC	1	15	1
+Virus/−MMC	0	30	3
−Virus/+MMC	0	8	0
+Virus/+MMC	0	25	2
B. Patient 2			
−Virus/−MMC	0	0	0
+Virus/−MMC	4	24	16
C. Patient 3			
−Virus/−MMC	10	40	7
−Virus/+MMC	0	6	0
+FAA virus/−MMC	26	115	12
+FAA virus/+MMC	14	50	8
+FAC virus/−MMC	26	46	6
+FAC virus/+MMC	1	10	0
D. Umbilical cord blood			
−Virus/−MMC	20	52	16
−Virus/+MMC	22	48	14

* The number of progenitor colonies (>50 cells/colony) measured at day 15 in methylcellulose culture. Results are expressed as colony no./2 × 10^4 $CD34^+$ cells plated. Samples were performed in duplicate and results are expressed as mean colony number. The MMC concentration was 1.0 nmol/L in all experiments.

DISCUSSION

We report here the functional complementation of FA-A hematopoietic cells using two amphotropic retroviral vector constructs. Phenotypic correction was determined by three different functional assays, namely, the resistance to MMC-induced cell death, insusceptibility to chromosomal breakage, and the restoration of normal cell-cycle kinetics. Complementation and correction occurred in four FA-A lymphoblastoid cell lines. Stable maintenance of the provirus was determined in all cases. Complementation of FA-A cells with these viral vectors has implications for the diagnosis, treatment, and pathophysiology of FA.

The *FAA* cDNA is 5.5 kb in length, making it too large for small viral vectors such as the adeno-associated virus (AAV). Consequently, we tested truncation mutants of *FAA* for the ability to complement. The 4.3-kb *FAA* fragment leads to an incomplete open reading frame that lacks 32 amino acids from the carboxyl-terminus. Unexpectedly, this fragment complemented HSC 72 and two other FA-A lymphoblastoid cell lines (K. Fu, unpublished data), suggesting that this shorter fragment of *FAA* may be functional.

Phenotypic complementation using recombinant viral vectors may be useful in assigning patients to either the FA-A or FA-C complementation group (collectively constitute nearly 80% of FA cases in the European and North American registries). For example, a D195V polymorphism in *FAC* was identified in the cell line EUFA 123.[21] We were able to demonstrate retroviral complementation using an *FAA* vector but not an *FAC* vector, confirming that the line belongs to the FA-A group (K.L. Fu et al, submitted). Group assignment using this approach was applied here to an unclassified patient (no. 3). Progenitor growth improved dramatically only following FAA transduction. This result not only validates

our FAA vector but suggests that retroviral gene transfer can assign patients to a FA complementation group.

Recently, immune reactivity to the neomycin phosphotransferase gene product has been described in patients who underwent cell transduction with viral vectors containing Neo^R.[22] Removal of the Neo^R gene from amphotropic retroviral vectors therefore may be desirable. In this study we used the unique characteristics of the *FAA* gene to generate complemented cells by selection solely on the basis of resistance to MMC-induced death. In contrast to our previous work (which used neomycin-selected stable transfectants), we used the inherent selection property of the *FAA* gene as the determinant of retroviral transduction.

The rescue and growth of transformed lymphoblasts and primary hematopoietic cells further support our hypothesis that restoration of corrected FA genes provides a selective advantage over mutant cells. Improved FA cell viability after transduction may result from resistance to apoptosis. Recent reports indicate that FA-C mutant cells are hypersensitive to apoptosis, and that complementation with the normal *FAC* cDNA confers resistance to apoptosis.[23,24] Hematopoietic progenitor cells from *fac* knock-out mice are hypersensitive to interferon-γ (IFN-γ), possibly through apoptotic mechanisms. Conversely, overexpression of *FAC* cDNA in hematopoietic cells of transgenic mice may confer resistance to Fas-mediated apoptosis.[25]

Rare patients with Fanconi anemia are phenotypically mosaic, meaning that one population of cells from such a patient is hypersensitive to DNA cross-linking agents while another subpopulation is normally resistant.[26] Analysis of mosaicism in FA patients indicates that spontaneous intragenic mitotic recombination and/or gene conversion occurred, resulting in the loss of one pathogenic mutation; this genetic reversion may lead to an improved phenotype.[27] Spontaneous reversion to normal from an inherited mutation has been observed in vivo in FA patients, with apparently improved hematopoiesis. Clonal hematopoiesis in these patients suggests that reversion occurred in single hematopoietic stem cell. Phenotypic reversion is not unique to FA but has also been reported in patients with adenosine deaminase deficiency[28] and ataxia telangiectasia.[29] This observation argues that FA gene-corrected cells may have a selective advantage in vivo.

Currently, a trial of hematopoietic progenitor cell transduction is being conducted at the Clinical Center of the National Institutes of Health for FA-C patients lacking a compatible BMT donor. The study thus far has confirmed that transfer of the *FAC* cDNA to multipotential progenitor cells is possible. Function of the wild-type *FAC* cDNA is suggested by the marked increase in progenitor numbers and MMC-resistant colonies with successive cycles of gene transduction in the three patients studied thus far. Coincident with this expansion in progenitor growth, two of the patients also had transient improvement in BM cellularity. These findings are encouraging regarding the potential utility of FA gene transfer in amelioration of the hematopoietic pathology. The *FAA* retroviral vectors described here should be evaluable in clinical trials designed on the basis of information learned from the FA-C trial.

ACKNOWLEDGMENT

We thank Dr Larry Arnold of the UNC Flow Cytometry and Janice Coleman of the UNC Cytogenetics Lab for their valued technical assistance. We thank Dr James Granfortuna (Moses Cone Hospital), Dr Sherri Zimmerman (Duke University Medical Center), and Dr John Bergsagel (Scottish Rite Children's Medical Center) for their assistance. We also thank Dr R. Jude Samulski and the UNC Gene Therapy Center for technical and material assistance. We thank the editorial assistance of Lee Hodge in preparing this paper.

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1: D86115. Homo sapiens gene...[gi:2244724]


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REFERENCE   1  (bases 1 to 90)
  AUTHORS   Shimizu,N.
  TITLE     Direct Submission
  JOURNAL   Submitted (19-JUN-1996) Nobuyoshi Shimizu, Keio University School
            of Medicine, Department of Molecular Biology; 35 Shinanomachi,
            Shinjuku-ku, Tokyo 160, Japan (E-mail:shimizu@dmb.med.keio.ac.jp,
            Tel:03-3351-2370, Fax:03-3351-2370)
REFERENCE   2  (sites)
  AUTHORS   Nagamine,K., Kudoh,J., Asakawa,S., Abe,I., Maeda,H., Tsujimoto,S.,
            Minoshima,S., Ito,F. and Shimizu,N.
  TITLE     Localization of 22 Exons to a 450-kb Region Involved in the
            Autoimmune Polyglandular Disease Type I (APECED) on Choromosome
            21q22.3
  JOURNAL   Unpublished
REFERENCE   3  (sites)
  AUTHORS   Kudoh,J., Nagamine,K., Asakawa,S., Abe,I., Kawasaki,K., Maeda,H.,
            Tsujimoto,S., Minoshima,S., Ito,F. and Shimizu,N.
  TITLE     Localization of 16 exons to a 450-kb region involved in the
            autoimmune polyglandular disease type I (APECED) on human
            chromosome 21q22.3
  JOURNAL   DNA Res. 4 (1), 45-52 (1997)
  MEDLINE   97323005
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                     /clone_lib="chromosome 21-specific cosmid library"
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     exon            1..90
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☐ 1: X56687. H.sapiens mRNA fo...[gi:28970] Related Sequences, OMIM, Protein, PubMed, Taxonomy, LinkOut

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LOCUS       HSAUTNOR     2489 bp    mRNA            PRI       03-APR-1997
DEFINITION  H.sapiens mRNA for autoantigen NOR-90.
ACCESSION   X56687
VERSION     X56687.1  GI:28970
KEYWORDS    NOR-90 autoantigen.
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 2489)
  AUTHORS   Chan,E.K.L.
  TITLE     Direct Submission
  JOURNAL   Submitted (15-NOV-1990) Chan E.K.L., Scripps Clinic & Research
            Foundation, 10666 N Torrey Pines Road, La Jolla CA 92037, U S A
REFERENCE   2  (bases 1 to 2489)
  AUTHORS   Chan,E.K., Imai,H., Hamel,J.C. and Tan,E.M.
  TITLE     Human autoantibody to RNA polymerase I transcription factor hUBF.
            Molecular identity of nucleolus organizer region autoantigen NOR-90
            and ribosomal RNA transcription upstream binding factor
  JOURNAL   J. Exp. Med. 174 (5), 1239-1244 (1991)
  MEDLINE   92044316
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                     /cell_line="HepG2 and MOLT-4"
                     /cell_type="liver and T cell origin"
                     /clone_lib="gZap, HepG2 cDNA and ggt11 MOLT-4 cDNA"
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                     PELSESELTRLLARMWNDLSEKKKAKYKAREAALKAQSERKPGGEREERGKLPESPKR
                     AEEIWQQSVIGDYLARFKNDRVKALKAMEMTWNNMEKKEKLMWIKKAAEDQKRYEREL
                     SEMRAPPAATNSSKKMKFQGEPKKPPMNGYQKFSQELLSNGELNHLPLKERMVEIGSR
                     WQRISQSQKEHYKKLAEEQQKQYKVHLDLWVKSLSPQDRAAYKEYISNKRKSMTKLRG
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[redacted]

BASE COUNT 704 a 668 c 740 g 377 t
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     121 ggctttgacc ccgggttgcc cggccagcac gaccgaggag gtggctggac agctggagga
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     661 atattcagga cttccagaga gagaaacagg agttcgagcg aaacctggcc cgattcaggg
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    1561 gcgactacct ggcccgcttc aagaatgacc gggtgaaggc cttgaaagcc atggaaatga
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    1681 aaaagcgata tgagagagag ctgagtgaga tgcgggcacc tccagctgct acaaattctt
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1: U26593. Human clone 61.1 ...[gi:1674389] Related Sequences, OMIM, Protein, PubMed, Taxonomy

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LOCUS       HSU26593      592 bp    DNA             PRI       18-NOV-1996
DEFINITION  Human clone 61.1 diabetes mellitus type I autoantigen (ICAp69)
            gene, partial cds.
ACCESSION   U26593
VERSION     U26593.1  GI:1674389
KEYWORDS    .
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 592)
  AUTHORS   Pietropaolo,M., Castano,L., Babu,S., Buelow,R., Kuo,Y.-L.S.,
            Martin,S., Martin,A., Powers,A.C., Prochazka,M., Naggert,J.,
            Leiter,E.H. and Eisenbarth,G.S.
  TITLE     Islet cell autoantigen 69 kD (ICA69). Molecular cloning and
            characterization of a novel diabetes-associated autoantigen
  JOURNAL   J. Clin. Invest. 92 (1), 359-371 (1993)
  MEDLINE   93315668
REFERENCE   2  (bases 1 to 592)
  AUTHORS   Miyazaki,I., Gaedigk,R., Hui,M.F., Cheung,R.K., Morkowski,J.,
            Rajotte,R.V. and Dosch,H.M.
  TITLE     Cloning of human and rat p69 cDNA, a candidate autoimmune target in
            type 1 diabetes
  JOURNAL   Biochim. Biophys. Acta 1227 (1-2), 101-104 (1994)
  MEDLINE   95002197
REFERENCE   3  (bases 1 to 592)
  AUTHORS   Gaedigk,R., Duncan,A.M., Miyazaki,I., Robinson,B.H. and Dosch,H.M.
  TITLE     ICA1 encoding p69, a protein linked to the development of type 1
            diabetes, maps to human chromosome 7p22
  JOURNAL   Cytogenet. Cell Genet. 66 (4), 274-276 (1994)
  MEDLINE   94215321
REFERENCE   4  (bases 1 to 592)
  AUTHORS   Miyazaki,I., Cheung,R.K., Gaedigk,R., Hui,M.F., Van der Meulen,J.,
            Rajotte,R.V. and Dosch,H.M.
  TITLE     T cell activation and anergy to islet cell antigen in type I
            diabetes
  JOURNAL   J. Immunol. 154 (3), 1461-1469 (1995)
  MEDLINE   95123099
REFERENCE   5  (bases 1 to 592)
  AUTHORS   Karges,W.J.P., Gaedigk,R., Hui,M.F., Cheung,R.K. and Dosch,H.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-MAY-1995) Hans-Michael Dosch, Immunology and Cancer,
            The Hospital for Sick Children, 555 University Ave., Toronto,
            Ontario M5G 1X8, Canada
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      181 gcctgttcac tttttaaact ttatttggaa tatttcttcc agtgttactt agctatattg
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☐ 1: X54162. Human mRNA for a ...[gi:28968] Related Sequences, OMIM, Protein, PubMed, Taxonomy, LinkOut

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LOCUS       HSAUTAN64     3881 bp    mRNA            PRI       25-JUL-1991
DEFINITION  Human mRNA for a 64 Kd autoantigen expressed in thyroid and
            extra-ocular  muscle.
ACCESSION   X54162
VERSION     X54162.1  GI:28968
KEYWORDS    64 Kd autoantigen; autoantigen.
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 3881)
  AUTHORS   Dong,Q.H.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-JUL-1990) Dong Q.H., Institute of Interdisciplinary
            Research, School of Medicine, Universite Libre de Bruxelles, Campus
            Erasme, 808 Route de Lennik, 1070 Brussels, Belgium
REFERENCE   2  (bases 1 to 3881)
  AUTHORS   Dong,Q., Ludgate,M. and Vassart,G.
  TITLE     Cloning and sequencing of a novel 64-kDa autoantigen recognized by
            patients with autoimmune thyroid disease
  JOURNAL   J. Clin. Endocrinol. Metab. 72 (6), 1375-1381 (1991)
  MEDLINE   91225220
FEATURES             Location/Qualifiers
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BASE COUNT 1065 a 1030 c 1084 g 702 t
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3361 ctcaacagca ggaggcacag cagtttcacc atctttgagg tgagggtggg gtgccccagc
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NCBI Sequence Viewer

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☐ 1: X16163. Human mRNA for Sm... [gi:36494]

Related Sequences, OMIM, Protein, PubMed, Taxonomy, LinkOut

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LOCUS       HSSMB          713 bp    mRNA            PRI       19-JUL-1995
DEFINITION  Human mRNA for SmB/B' autoimmune antigene (K5-1).
ACCESSION   X16163
VERSION     X16163.1  GI:36494
KEYWORDS    autoimmune antigen; systemic lupus erythematosus.
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 713)
  AUTHORS   Sharpe,N.G., Williams,D.G., Howarth,D.N., Coles,B. and
            Latchman,D.S.
  TITLE     Isolation of cDNA clones encoding the human Sm B/B' auto-immune
            antigen and specifically reacting with human anti-Sm auto-immune
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  JOURNAL   FEBS Lett. 250 (2), 585-590 (1989)
  MEDLINE   89325637
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NCBI Sequence Viewer

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☐ 1: X69936. H.sapiens mRNA fo...[gi:31757] Related Sequences, Protein, PubMed, Taxonomy, LinkOut

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DEFINITION  H.sapiens mRNA for glutamate decarboxylase.
ACCESSION   X69936
VERSION     X69936.1  GI:31757
KEYWORDS    autoantigen; glutamate decarboxylase; glutamic acid decarboxylase;
            histidine-hexapeptide fusion protein.
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 1803)
  AUTHORS   Northemann,W.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-DEC-1992) W. Northemann, Elias Entwicklungslabor,
            Department of molecular Biology, Obere Hardtstrasse 18, D-7800
            Freiburg, FRG
REFERENCE   2  (bases 1 to 1803)
  AUTHORS   Mauch,L., Abney,C.C., Berg,H., Scherbaum,W.A., Liedvogel,B. and
            Northemann,W.
  TITLE     Characterization of a linear epitope within the human pancreatic
            64-kDa glutamic acid decarboxylase and its autoimmune recognition
            by sera from insulin-dependent diabetes mellitus patients
  JOURNAL   Eur. J. Biochem. 212 (2), 597-603 (1993)
  MEDLINE   93185681
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1: J04977. Human Ku autoimmu...[gi:186791] Related Sequences, OMIM, Protein, PubMed, Taxonomy, LinkOut

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DEFINITION  Human Ku autoimmune antigen gene, complete cds.
ACCESSION   J04977
VERSION     J04977.1  GI:186791
KEYWORDS    Ku antigen; nonhistone DNA binding protein; nuclear protein.
SOURCE      Human fetal liver, cDNA to mRNA.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 3052)
  AUTHORS   Yaneva,M., Wen,J., Ayala,A. and Cook,R.
  TITLE     cDNA-derived amino acid sequence of the 86-kDa subunit of the Ku
            antigen
  JOURNAL   J. Biol. Chem. 264 (23), 13407-13411 (1989)
  MEDLINE   89340410
COMMENT     Draft entry and computer-readable sequence for [1] kindly submitted
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BASE COUNT      906 a    592 c    708 g    846 t
ORIGIN      58 bp upstream of PvuII site.
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☐ 1: J03798. Human autoantigen...[gi:338264] Related Sequences, OMIM, Protein, PubMed, Taxonomy, LinkOut

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LOCUS       HUMSNRNPD    1633 bp    mRNA            PRI       03-AUG-1993
DEFINITION  Human autoantigen small nuclear ribonucleoprotein Sm-D mRNA,
            complete cds.
ACCESSION   J03798
VERSION     J03798.1  GI:338264
KEYWORDS    autoantigen; ribonucleoprotein.
SOURCE      Human B-lymphocyte, cDNA to mRNA, clone D45-2.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 1633)
  AUTHORS   Rokeach,L.A., Haselby,J.A. and Hoch,S.O.
  TITLE     Molecular cloning of a cDNA encoding the human Sm-D autoantigen
  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 85, 4832-4836 (1988)
  MEDLINE   88263041
COMMENT     Draft entry and computer-readable sequence [1] kindly submitted by
            L.Rokeach 20-JUL-1988.
            The Sm-D protein coded by cDNA D45-2, being a snRNP, is evidently
            involved in the mRNA splicing of highter eukaryotes; in the
            autoimmune disease systemic lupus erythematosus, antinuclear
            antibodies are developed with Sm specificity.
FEATURES             Location/Qualifiers
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☐ 1: M24499. Human cytochrome ...[gi:522194] Related Sequences, Protein, PubMed, Taxonomy, LinkOut

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LOCUS       HUMP450DB1   1195 bp    mRNA            PRI       04-AUG-1994
DEFINITION  Human cytochrome P450db1 mRNA, 3' end, clone HLD8.2.
ACCESSION   M24499
VERSION     M24499.1  GI:522194
KEYWORDS    cytochrome P450db1.
SOURCE      Homo sapiens (individual_isolate Hepatitis patient) liver cDNA to
            mRNA.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 1195)
  AUTHORS   Manns,M.P., Johnson,E.F., Griffin,K.J., Tan,E.M. and Sullivan,K.F.
  TITLE     Major antigen of liver kidney microsomal autoantibodies in
            idiopathic autoimmune hepatitis is cytochrome P450db1
  JOURNAL   J. Clin. Invest. 83, 1066-1072 (1989)
  MEDLINE   89155788
COMMENT     On Aug 5, 1994 this sequence version replaced gi:341176.
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NCBI Sequence Viewer

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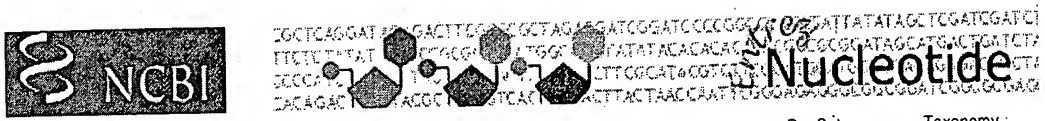

1: M28639. Human autoimmune ...[gi:291864]

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ACCESSION   M28639
VERSION     M28639.1  GI:291864
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SOURCE      Homo sapiens Graves' thyroid cDNA to mRNA.
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REFERENCE   1  (bases 1 to 572)
  AUTHORS   Hirayu,H., Seto,P., Magnusson,R.P., Filetti,S. and Rapoport,B.
  TITLE     Molecular cloning and partial characterization of a new autoimmune
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  JOURNAL   J. Clin. Endocrinol. Metab. 64, 578-584 (1984)
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1: J Immunol Methods 1995 Jul 17;184(1):81-9 Related Articles, Books, LinkOut

Isolation of human blood dendritic cells by discontinuous Nycodenz gradient centrifugation.

McLellan AD, Starling GC, Hart DN.

Haematology/Immunology Research Laboratory, Christchurch Hospital, New Zealand.

The most potent antigen presenting cell present in peripheral blood, lymphoid and non-lymphoid tissue is the dendritic cell (DC). The study of human DC has been restricted by their low frequency in the tissues and the lack of a truly DC specific surface marker to assist in identification and isolation. Standard techniques for the isolation of blood DC generally employ a period of in vitro culture followed by flotation on dense albumin gradients, or more recently, discontinuous gradients of metrizamide. Dense albumin gradients are time consuming to prepare, giving low and variable yields of DC. Metrizamide is more convenient, although exposure of monocytes to metrizamide can decrease the expression of CD14 and alter the accessory cell properties of antigen presenting cells. Here we demonstrate that Nycodenz gradient centrifugation of 16 h cultured, T lymphocyte depleted, peripheral blood mononuclear cells (PBMC) reliably yields a population of low density cells that is highly enriched for DC. Most B and residual T lymphocytes are depleted and NK cell numbers are reduced two-fold from the interface cell population. The high density pellet fraction exhibits very little allostimulatory activity, indicating that few DC pass into the pellet. The low density fraction contains a significant population (20 +/- 5 (SD)%, n = 8) of cells which fail to stain for the lineage markers CD3, CD11b, CD14, CD16, CD19 and CD57. Nycodenz exhibits low toxicity, does not alter the allostimulatory activity of antigen presenting cells, and is therefore ideal for the isolation of cultured DC.

PMID: 7622872 [PubMed - indexed for MEDLINE]

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